(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 January 2001 (11.01.2001)

PCT

(10) International Publication Number
WO 01/02574 A1

(51) International Patent Classification[7]: C12N 15/29, 15/82, A01H 5/00, 5/10

(21) International Application Number: PCT/US00/18270

(22) International Filing Date: 30 June 2000 (30.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/142,214 2 July 1999 (02.07.1999) US

(71) Applicant: **DUKE UNIVERSITY** [US/US]; 230 North Building, Research Drive, Durham, NC 27708 (US).

(72) Inventors: **DONG, Xinnian**; 3619 Dover Road, Durham, NC 27707 (US). **LI, Xin**; Apartment 201, 808 F Street, Davis, CA 95616 (US).

(74) Agent: **ELBING, Karen, L.**; Clark & Elbing LLP, 176 Federal Street, Boston, MA 02110-2214 (US).

(81) Designated States *(national)*: AU, CA, JP.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/02574 A1

(54) Title: NEGATIVE REGULATORS OF SYSTEMIC ACQUIRED RESISTANCE

(57) Abstract: The invention generally features a non-naturally occurring plant (e.g. a transgenic plant) having decreased expression of an endogenous negative regulator polypeptide of systemic acquired resistance (SAR), wherein the decreased expression renders the non-naturally occurring plant resistant to a plant pathogen.

NEGATIVE REGULATORS OF SYSTEMIC ACQUIRED RESISTANCE

Statement as to Federally Sponsored Research

This invention was made in part with Government funding and the Government therefore has certain rights in the invention. In particular, portions of the invention disclosed herein were funded by the United States Department of Agriculture and the National Science Foundation.

Background of the Invention

This application claims benefit of U.S. provisional application 60/142,214, filed on July 2, 1999.

Systemic acquired resistance ("SAR") is a general plant defense response that can be triggered after a local infection by a necrotizing pathogen. This response is long lasting and effective against a variety of pathogens including fungi, bacteria, and viruses (Ryals et al., *Plant Cell* 8:1809-1819, 1996). Induction of SAR is correlated with up-regulation of several well-characterized pathogenesis-related (*"PR"*) genes (Van Loon and Van Kammen, *Virology* 40:199-211, 1970; Ward et al., *Plant Cell* 3:1085-1094, 1991; Yalpani et al., *Plant Cell* 3:809-818, 1991; Uknes et al., *Plant Cell* 4:645-656, 1992). In particular, *PR-1* and β-1,3-glucanase (*"BGL2,"* also known as *PR-2*) have been widely used as molecular markers for SAR (Uknes et al., *Plant Cell* 4:645-656, 1992; Bowling et al., *Plant Cell* 6:1845-1857, 1994; Cao et al., *Plant Cell* 6:1583-1592, 1994). Convincing evidence has shown that salicylic acid ("SA") is a necessary and sufficient signal for SAR induction. The onset of SAR is accompanied by an increase in the endogenous levels of SA (Malamy et al., *Science* 250:1002-1004, 1990; Métraux et al., *Science* 250:1004-1006, 1990; Rasmussen et al., *Plant Physiol.* 97:1342-1347, 1991) and can also be brought on by exogenous application of SA (White, *Virology* 99:410-412, 1979) or its analogs such as 2,6-dichloroisonicotinic acid ("INA"; Métraux et al., In: *Advances in Molecular Genetics of Plant-Microbe Interactions,* Hennecke, H. and Verma D.P.S., eds.,

Kluwer Academic Publishers, Dordrecht, The Netherlands, Vol. 1, pp. 432-439. 1991) and benzo(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester ("BTH"; Görlach et al., *Plant Cell* 8:629-643, 1996). Furthermore, transgenic plants expressing the SA-degrading enzyme salicylate hydroxylase are unable to accumulate SA and are compromised in SAR (Gaffney et al., *Science* 261:754-756, 1993).

Several genetic screens have been conducted in *Arabidopsis* to identify the regulatory components of the signaling pathway leading to SAR. Among the SAR compromised mutants, *npr1s* (also known as *nim1s*) are the only mutants identified that affect a function downstream of the SA signal (Cao et al., *Plant Cell* 6:1583-1592, 1994; Delaney et al., *Proc. Natl. Acad. Sci. USA* 92:6602-6606, 1995; Glazebrook et al., *Genetics* 143:973-982, 1996; Ryals et al., *Plant Cell* 9:425-439, 1997; Shah et al., *Mol. Plant Microbe Interact.* 10:69-78, 1997). The *npr1* mutants are impaired in the ability to accumulate *PR* mRNA or to mount an SAR response in the presence of an SAR inducer. The *NPR1* gene encodes a novel protein containing at least four ankyrin repeats (Cao et al., *Cell* 88:57-63, 1997; Ryals et al., *Plant Cell* 9:425-439, 1997), which are found in proteins of diverse functions (Bork, *Proteins* 17:363-374, 1993), including IkB and Cactus that regulate animal immune responses (Thanos and Maniatis, *Cell* 80:529-532, 1995; Lemaitre et al., *Cell* 86:973-983, 1996). The functional importance of these ankyrin repeats in NPR1 has been clearly demonstrated by the isolation of several *npr1* mutants that contain lesions in the ankyrin repeat consensus (Cao et al., *Cell* 88:57-63, 1997; Ryals et al., *Plant Cell* 9:425-439, 1997). Specifically, in the *npr1-1* mutant, where a highly conserved histidine (residue 334) in the third ankyrin repeat is changed to a tyrosine, the SA- or INA-induced expression of a reporter gene *BGL2-GUS* and the endogenous *PR* genes is abolished (Cao et al., *Cell* 88:57-63, 1997).

Despite such progress, a need exists in the art for identifying other important genetic signaling components of SAR. Such components provide novel targets for developing not only crops having resistance to a broad spectrum of pathogens, but also for developing new crop protection methodologies.

Summary of the Invention

The present invention capitalizes on the discovery of a negative regulator polypeptide of systemic acquired resistance (SAR). Manipulation of the expression of this SAR component facilitates the production of plants having broad spectrum disease resistance.

Accordingly, the invention generally features a non-naturally occurring plant having decreased expression of an endogenous negative regulator polypeptide of SAR, wherein the decreased expression renders the non-naturally occurring plant resistant to a plant pathogen. Preferably, the non-naturally occurring plant is a transgenic plant or a mutant plant. In preferred embodiments, such a transgenic plant includes a transgene that, when expressed in the transgenic plant, silences gene expression of the endogenous negative regulator polypeptide of SAR.

In still other preferred embodiments, the transgenic plant includes a transgene that expresses an antisense molecule of the negative regulator polypeptide of SAR; a transgene that, when expressed in the transgenic plant, co-suppresses expression of the negative regulator polypeptide of SAR (such as a mutated form of the negative regulator polypeptide); or a transgene that encodes a dominant negative gene product; or any combination thereof.

In other preferred embodiments, the plant of the invention includes a point mutation, a deletion, or an insertion, or any combination thereof, in a gene that encodes the negative regulator polypeptide of SAR. Decreased expression of the negative regulator polypeptide of SAR in the plant of the invention may occur at the transcriptional, translational, post-transcriptional, or post-translational levels of gene expression.

In another aspect, the invention features an isolated nucleic acid molecule (for example, an isolated DNA molecule such as cDNA) including a sequence encoding a negative regulator polypeptide of SAR. In preferred embodiments, the isolated nucleic acid molecule includes a negative regulator polypeptide of SAR that is substantially identical to the amino acid sequence shown in Fig. 3A. An exemplary isolated nucleic acid molecule of the invention is shown in SEQ ID NO:1. In even

another embodiment, the negative regulator polypeptide of SAR has at least 30% identity to the amino acid sequence shown in Fig. 3A. Preferably, such a polypeptide is leucine-rich or has substantial identity to the N domain of a retinoblastoma protein or both. Such a polypeptide is shown in SEQ ID NO:2.

In other preferred embodiments, the isolated nucleic acid molecule of the invention includes a sequence that encodes a polypeptide that, when expressed in a cell of a plant, represses transcription or is substantially localized to the nucleus or both.

In yet other embodiments, the isolated nucleic acid molecule including a sequence encoding a negative regulator polypeptide of SAR hybridizes specifically to a nucleic acid molecule including the sequence of Fig. 3A or 3B.

In still other embodiments, the invention features the isolated nucleic acid molecule operably linked to an expression control region.

In related aspects, the invention features an expression vector including the isolated nucleic acid molecule of the invention and a cell (for example, a prokaryotic cell, such as *Agrobacterium* or *E. coli,* or a eukaryotic cell, such as a mammalian, insect, yeast, or plant cell) including the isolated nucleic acid molecule or vector.

In yet another aspect, the invention features a transgenic plant or transgenic plant component including a nucleic acid molecule of the invention, wherein the nucleic acid molecule is expressed in the transgenic plant or the transgenic plant component. Preferably, the transgenic plant or transgenic plant component is an angiosperm (for example, a monocot or dicot). In preferred embodiments, the transgenic plant or transgenic plant component is a cruciferous plant. The invention further includes a seed produced by the transgenic plant or transgenic plant component, or progeny thereof.

In still other related aspects, the invention features an isolated DNA molecule encoding an antisense RNA of a negative regulator polypeptide of SAR; an expression vector including the DNA molecule encoding the antisense RNA of a negative regulator of SAR; or a transgenic plant or transgenic plant component (or a seed or a cell thereof) that harbors the vector or the isolated DNA molecule encoding the

antisense RNA molecule.

In another aspect, the invention features a substantially pure negative regulator polypeptide of SAR. In preferred embodiments, the polypeptide includes an amino acid sequence that is substantially identical to the amino acid sequence of Fig. 3A or to the N domain of a retinoblastoma protein of Fig. 3C. In still other preferred embodiments, the polypeptide represses transcription, is substantially localized to the nucleus, or is leucine-rich, or has any combination of these characteristics. An exemplary negative regulator polypeptide is shown in SEQ ID NO:2.

In another aspect, the invention features a method of producing a negative regulator polypeptide of SAR, including the steps of: (a) providing a cell transformed with an isolated nucleic acid molecule of the invention; (b) culturing the transformed cell under conditions for expressing the isolated nucleic acid molecule; and (c) recovering the negative regulator polypeptide of SAR. The invention further includes a recombinant negative regulator polypeptide of SAR produced according to this method.

In another aspect, the invention features a substantially pure antibody (e.g. a polyclonal or monoclonal antibody) that specifically recognizes and binds to a negative regulator polypeptide of SAR. Preferably, such an antibody recognizes and binds to a recombinant negative regulator polypeptide of SAR.

In yet another aspect, the invention features methods of isolating a negative regulator gene of SAR or a fragment thereof. One such method includes the steps of: (a) contacting the nucleic acid molecule of Fig. 3A or a portion thereof with a nucleic acid preparation from a plant cell under hybridization conditions providing detection of nucleic acid sequences having at least 30% or greater sequence identity to the nucleic acid sequence of Fig. 3A; and (b) isolating the hybridizing nucleic acid sequences.

A second method of the invention includes the steps of: (a) providing a sample of plant cell DNA; (b) providing a pair of oligonucleotides having sequence identity to a region of the nucleic acid molecule of Fig. 3A; (c) contacting the pair of oligonucleotides with the plant cell DNA under conditions suitable for polymerase

chain reaction-mediated DNA amplification; and (d) isolating the amplified negative regulator gene of SAR or fragment thereof. In preferred embodiments, the amplification step is carried out using a sample of cDNA prepared from a plant cell; and the negative regulator gene of SAR encodes a polypeptide which is at least 30% identical to the amino acid sequence of Fig. 3A.

Further, the invention features several gene silencing methods for conferring disease resistance on a plant. Such methods include, without limitation, co-suppression and antisense technologies, expression of altered or mutated forms of the negative regulator polypeptide, as well as the expression of dominant negative forms of the negative regulator. Silencing the expression of the negative regulator provides a plant or plant component of the invention with broad spectrum disease resistance to plant pathogens including, but not limited to, those described herein.

In yet another aspect, the invention features a method for conferring disease resistance on a transgenic plant cell. The method includes reducing the level of an endogenous negative regulator polypeptide of SAR in a transgenic plant cell, a transgenic plant, or a transgenic plant component. In preferred embodiments, methods for reducing the level of the endogenous negative regulator polypeptide of SAR include expressing a transgene encoding an antisense nucleic acid molecule of a negative regulator gene of SAR in the transgenic plant cell. In other preferred embodiments, reducing the level of the endogenous negative regulator polypeptide of SAR includes co-suppression of the endogenous negative regulator gene of SAR in the transgenic plant cell. Still another method for reducing the level of the endogenous negative regulator polypeptide of SAR includes expressing a dominant negative gene product (for example, a mutated form of the endogenous negative regulator polypeptide of SAR) in the transgenic plant cell, the transgenic plant, or the transgenic plant component. And in still other preferred embodiments, the expression of altered or mutant forms of the negative regulators polypeptide may be used to confer disease resistance. Plants of the invention may be regenerated from any of these plant cells according to standard methods known in the art.

In yet another aspect, the invention features a method for identifying a compound that modulates the expression of a gene encoding a negative regulator polypeptide of SAR. The method includes the steps of: (a) providing a cell (for example, a prokaryotic cell, such as *E. coli*, or a eukaryotic cell, such as a mammalian, yeast, insect cell, or plant cell) including a gene encoding a negative regulator polypeptide of SAR; (b) applying to the cell a candidate compound; and (c) measuring expression of the gene encoding the negative regulator polypeptide of SAR, an increase or decrease in expression relative to an untreated control sample being an indication that the compound modulates expression of the negative regulator polypeptide of SAR. In preferred embodiments, the gene encodes a negative regulator polypeptide of SAR that is substantially identical to the amino acid sequence shown in Fig. 3A. In other preferred embodiments, the compound decreases the expression of the gene that encodes the negative regulator polypeptide of SAR. In yet other preferred embodiments, the compound increases the expression of the gene that encodes the negative regulator polypeptide of SAR. Compounds identified using these methods are useful as crop protectants.

In another aspect, the invention features a method for identifying a compound that modulates the activity of a negative regulator polypeptide of SAR in a plant or plant component. In general, the method includes the steps of: (a) providing a plant or plant component expressing an isolated DNA molecule encoding a negative regulator polypeptide of SAR; (b) applying to the plant or plant component a candidate compound; and (c) measuring the activity of the negative regulator polypeptide of SAR, an increase or decrease in activity relative to an untreated control sample being an indication that the compound modulates activity of the negative regulator polypeptide of SAR. In preferred embodiments, the gene encodes a negative regulator polypeptide of SAR that is substantially identically to the amino acid sequence shown in Fig. 3A. In another preferred embodiment, the compound decreases the activity of the negative regulator polypeptide of SAR. In another embodiment, the compound increases the activity of the negative regulator polypeptide of SAR.

In another aspect, the invention features a method for increasing the resistance of a plant or plant component to a pathogen, involving applying a compound to the plant or plant component that decreases the expression of a gene encoding a negative regulator polypeptide of SAR.

In another aspect, the invention features a method for increasing the resistance of a plant or plant component to a pathogen, involving applying a compound to the plant or plant component that decreases activity of a negative regulator polypeptide of SAR.

In yet another aspect, the invention features a method for increasing the resistance of a plant or plant component to a pathogen, involving applying a chemical regulator of SAR to a plant or plant component that has decreased expression of a negative regulator polypeptide of SAR. In preferred embodiments, the chemical inducer of SAR is SA, INA, or BTH, or any combination thereof.

By "negative regulator polypeptide of SAR" is meant a polypeptide which represses a plant's systemic acquired resistance. An exemplary negative regulator polypeptide of SAR, SNI1, from *Arabidopsis* is described herein. Additional negative regulator polypeptides may be identified and isolated from any plant species, especially agronomically important crop plants, using any of the sequences disclosed herein in combination with conventional methods known in the art.

By "gene silencing" is meant a decrease in the level of gene expression (for example, expression of a gene encoding a negative regulator polypeptide of SAR (for example, *SNI1*)) by at least 30-50%, preferably by at least 50-80%, and more preferably by at least 80-95% or greater relative to the level in a control plant (for example, a wild-type plant). Reduction of such expression levels may be accomplished by employing standard methods which are known in the art including, without limitation, antisense and co-suppression technologies, expression of a dominant negative gene product, or through the generation of mutated genes using standard mutagenesis techniques. Levels of negative regulator polypeptide or transcript are monitored according to any standard technique including, but not limited to, northern blotting, RNase protection, or immunoblotting.

By "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (for example, glycosylation or phosphorylation).

By "substantially identical" is meant a polypeptide or nucleic acid exhibiting at least 20%, preferably 50%, more preferably 80%, and most preferably 90%, or even 95% or greater identity to a reference amino acid sequence (for example, the amino acid sequence shown in Fig. 3A or to an N domain (e.g., the N domain of retinoblastoma shown in Fig. 3C)) or nucleic acid sequence (for example, the nucleic acid sequences shown in Fig. 3A or Fig. 3B). For polypeptides, the length of comparison sequences will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids or greater. For nucleic acids, the length of comparison sequences will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides of greater.

Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705, BLAST, FASTA, PILEUP/PRETTYBOX programs, or other publicly available sequence analysis programs, for example those found at http://www.expasy.ch/tools). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By a "substantially pure polypeptide" is meant a negative regulator polypeptide of SAR (for example, an SNI polypeptide such as SNI1) that has been separated from components which naturally accompany it. Typically, the polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most

preferably at least 99%, by weight, a negative regulator polypeptide of SAR. A substantially pure negative regulator polypeptide of SAR may be obtained, for example, by extraction from a natural source (for example, a plant cell); by expression of a recombinant nucleic acid encoding a negative regulator polypeptide of SAR; or by chemically synthesizing the protein. Purity can be measured by any appropriate method, for example, column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

By "isolated DNA" is meant DNA that is free of the genes which, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or that exists as a separate molecule (for example, a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

By "derived from" is meant isolated from or having the sequence of a naturally-occurring sequence (for example, a cDNA, genomic DNA, synthetic DNA, or combination thereof).

By "specifically hybridizes" is meant that a nucleic acid sequence is capable of hybridizing to a DNA sequence at least under low stringency conditions as described herein, and preferably under high stringency conditions, also as described herein. These conditions are well known in the art and may be altered in order to identify or detect identical or related polynucleotide sequences.

By "transformed cell" is meant a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule, for example, a DNA molecule encoding a negative regulator polypeptide of SAR.

By "positioned for expression" is meant that the DNA molecule is positioned adjacent to a DNA sequence which directs transcription (i.e., facilitates the production of an RNA molecule) and, if a protein product is desired, translation of the sequence

(i.e., facilitates the production of, for example, a negative regulator polypeptide of SAR or a recombinant protein).

By "reporter gene" is meant a gene whose expression may be assayed; such genes include, without limitation, β-glucuronidase (GUS), luciferase, chloramphenicol transacetylase (CAT), green fluorescent protein (GFP), β-galactosidase, herbicide resistant genes, and antibiotic resistance genes.

By "expression control region" is meant any minimal sequence sufficient to direct transcription. Included in the invention are promoter elements that are sufficient to render promoter-dependent gene expression controllable for cell-, tissue-, or organ-specific gene expression, or elements that are inducible by external signals or agents (for example, light-, pathogen-, wound-, stress-, or hormone-inducible elements or chemical inducers such as SA or INA); such elements may be located in the 5' or 3' regions of the native gene or engineered into a transgene construct.

By "operably linked" is meant that a gene and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (for example, transcriptional activator proteins) are bound to the regulatory sequence(s).

By "plant cell" is meant any self-propagating cell bounded by a semi-permeable membrane and containing a plastid. Such a cell also requires a cell wall if further propagation is desired. Plant cell, as used herein includes, without limitation, algae, cyanobacteria, seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores.

By "plant component" is meant a part, segment, or organ obtained from an intact plant or plant cell. Exemplary plant components include, without limitation, somatic embryos, leaves, stems, roots, flowers, tendrils, fruits, scions, and rootstocks.

By "crucifer" is meant any plant that is classified within the Cruciferae family. The Cruciferae include many agricultural crops, including, without limitation, rape (for example, *Brassica campestris* and *Brassica napus*), broccoli, cabbage, brussel sprouts, radish, kale, Chinese kale, kohlrabi, cauliflower, turnip, rutabaga, mustard,

horseradish, and *Arabidopsis*.

By "transgene" is meant any piece of DNA which is inserted by artifice into a cell, and becomes part of the genome of the organism which develops from that cell. Such a transgene may include a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene having sequence identity to an endogenous gene of the organism.

By "transgenic" is meant any cell which includes a DNA sequence which is inserted by artifice into a cell and becomes part of the genome of the organism which develops from that cell. As used herein, the transgenic organisms are generally transgenic plants and the DNA (for example, a transgene) is inserted by artifice into the nuclear or plastidic genome. A transgenic plant according to the invention may contain one or more of the nucleic acid molecules described herein

By "pathogen" is meant an organism whose infection of viable plant tissue elicits a disease response in the plant tissue. Such pathogens include, without limitation, bacteria, mycoplasmas, fungi, oomycetes, insects, nematodes, viruses, and viroids. Examples of such plant diseases caused by these pathogens are described in Chapters 11-16 of Agrios, *Plant Pathology*, 3rd ed., Academic Press, Inc., New York, 1988.

Examples of bacterial pathogens include, without limitation, *Erwinia* (for example, *E. carotovora*), *Pseudomonas* (for example, *P. syringae*), and *Xanthomonas* (for example, *X. campepestris* and *X. oryzae*).

Examples of fungal or fungal-like disease-causing pathogens include, without limitation, *Alternaria* (for example, *A. brassicola* and *A.solani*), *Ascochyta* (for example, *A. pisi*), *Botrytis* (for example, *B. cinerea*), *Cercospora* (for example, *C. kikuchii* and *C. zaea-maydis*), *Colletotrichum sp.* (for example, *C. lindemuthianum*), *Diplodia* (for example, *D. maydis*), *Erysiphe* (for example, *E. graminis f.sp. graminis* and *E. graminis f.sp. hordei*), *Fusarium* (for example, *F. nivale* and *F. oxysporum*, *F. graminearum*, *F. solani*, *F. monilforme*, and *F. roseum*), *Gaeumanomyces* (for example, *G. graminis f.sp. tritici*), *Helminthosporium* (for example, *H. turcicum*, *H. carbonum*, and *H. maydis*), *Macrophomina* (for example, *M. phaseolina* and

Maganaporthe grisea), *Nectria* (for example, *N. heamatocacca*), *Peronospora* (for example, *P. manshurica*, *P. tabacina*), *Phoma* (for example, *P. betae*), *Phymatotrichum* (for example, *P. omnivorum*), *Phytophthora* (for example, *P. cinnamomi*, *P. cactorum*, *P. phaseoli*, *P. parasitica*, *P. citrophthora*, *P. megasperma f.sp. sojae*, and *P. infestans*), *Plasmopara* (for example, *P. viticola*), *Podosphaera* (for example, *P. leucotricha*), *Puccinia* (for example, *P. sorghi*, *P. striiformis*, *P. graminis f.sp. tritici*, *P. asparagi*, *P. recondita*, and *P. arachidis*), *Puthium* (for example, *P. aphanidermatum*), *Pyrenophora* (for example, *P. tritici-repentens*), *Pyricularia* (for example, *P. oryzea*), *Pythium* (for example, *P. ultimum*), *Rhizoctonia* (for example, *R. solani* and *R. cerealis*), *Scerotium* (for example, *S. rolfsii*), *Sclerotinia* (for example, *S. sclerotiorum*), *Septoria* (for example, *S. lycopersici*, *S. glycines*, *S. nodorum* and *S. tritici*), *Thielaviopsis* (for example, *T. basicola*), *Uncinula* (for example, *U. necator*), *Venturia* (for example, *V. inaequalis*), and *Verticillium* (for example, *V. dahliae* and *V. albo-atrum*).

Examples of pathogenic nematodes include, without limitation, root-knot nematodes (for example, *Meloidogyne sp.* such as *M. incognita*, *M. arenaria*, *M. chitwoodi*, *M. hapla*, *M. javanica*, *M. graminocola*, *M. microtyla*, *M. graminis*, and *M. naasi*), cyst nematodes (for example, *Heterodera sp.* such as *H. schachtii*, *H. glycines*, *H. sacchari*, *H. oryzae*, *H. avenae*, *H. cajani*, *H. elachista*, *H. goettingiana*, *H. graminis*, *H. mediterranea*, *H. mothi*, *H. sorghi*, and *H. zeae,* or, for example, *Globodera sp.* such as *G. rostochiensis* and *G. pallida*), root-attacking nematodes (for example, *Rotylenchulus reniformis*, *Tylenchuylus semipenetrans*, *Pratylenchus brachyurus*, *Radopholus citrophilus*, *Radopholus similis*, *Xiphinema americanum*, *Xiphinema rivesi*, *Paratrichodorus minor*, *Heterorhabditis heliothidis*, and *Bursaphelenchus xylophilus*), and above-ground nematodes (for example, *Anguina funesta*, *Anguina tritici*, *Ditylenchus dipsaci*, *Ditylenchus myceliphagus*, and *Aphenlenchoides besseyi*).

Examples of viral pathogens include, without limitation, tobacco mosaic virus (TMV), tobacco necrosis virus (TNV), potato leaf roll virus, potato virus X, potato virus Y, tomato spotted wilt virus, and tomato ring spot virus.

By "increased level of resistance" is meant a level of resistance to a disease-causing pathogen in a non-naturally occurring plant (or cell or seed thereof) which is greater than the level of resistance in a control plant (for example, a non-transgenic plant or wild-type). In preferred embodiments, the level of resistance in a non-naturally occurring transgenic plant of the invention is at least 5% to 20% (and preferably 30% or 40%) greater than the resistance exhibited by a control plant. In other preferred embodiments, the level of resistance to a disease-causing pathogen is 50% greater, 60% greater, and more preferably even 75% or 90% greater than a control plant; with up to 100% or greater above the level of resistance as compared to a control plant being most preferred. The level of resistance is measured using conventional methods. For example, the level of resistance to a pathogen may be determined by comparing physical features and characteristics (for example, plant height and weight) or by comparing disease symptoms (for example, delayed lesion development, reduced lesion size, leaf wilting and curling, water-soaked spots, amount of pathogen growth, and discoloration of cells) of the non-naturally occurring plant (e.g., a transgenic plant).

By "detectably-labeled" is meant any direct or indirect means for marking and identifying the presence of a molecule, for example, an oligonucleotide probe or primer, a gene or fragment thereof, or a cDNA molecule or a fragment thereof. Methods for detectably-labeling a molecule are well known in the art and include, without limitation, radioactive labeling (for example, with an isotope such as ^{32}P or ^{35}S) and nonradioactive labeling (for example, fluorescence of chemiluminescent labeling, for example, fluorescein labeling).

As discussed above, a gene encoding a negative regulator polypeptide of SAR has been identified. Accordingly, the invention provides a number of important advances and advantages for the protection of plants against pathogens. For example, by reducing the expression of such negative regulators of SAR in a plant, the invention facilitates an effective and economical means for in-plant protection against plant pathogens. Such protection against pathogens reduces or minimizes the need for traditional chemical practices (for example, application of fungicides, bactericides,

nematicides, insecticides, or viricides) that are typically used by farmers for controlling the spread of plant pathogens and providing protection against disease-causing pathogens. In addition, because plants having decreased expression of a negative regulator of SAR are less vulnerable to pathogens and their diseases, the invention further provides for increased production efficiency, as well as for improvements in quality and yield of crop plants and ornamentals. Thus, the invention contributes to the production of high quality and high yield agricultural products, for example, fruits, ornamentals, vegetables, cereals and field crops having reduced spots, blemishes, and blotches that are caused by pathogens; agricultural products with increased shelf-life and reduced handling costs; and high quality and yield crops for agricultural (for example, cereal and field crops), industrial (for example, oilseeds), and commercial (for example, fiber crops) purposes. Furthermore, because the invention reduces the necessity for chemical protection against plant pathogens, the invention benefits the environment where the crops are grown. Genetically-improved seeds and other plant products that are produced using plants expressing the genes described herein also render farming possible in areas previously unsuitable for agricultural production.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Detailed Description of the Invention

The drawings will first be described.

Drawings

FIGURE 1A shows the expression of the *BGL2-GUS* reporter gene in wild type (WT), *npr1-1, snil npr1-1*, and *snil NPR1* plants. Two-week-old seedlings grown on MS (Murashige-Skoog) media in the presence or absence of INA (0.1 mM) were stained for GUS activity according to the methods described by Jefferson et al. (*EMBO J.* 6:3901-3907, 1987).

FIGURE 1B shows an RNA blot analysis of *PR-1* gene expression in wild type (WT), *npr1-1, snil npr1-1*, and *snil NPR1* plants. RNA samples were prepared from 2-week-old seedlings grown on MS media or MS with 0.1 mM INA. For each sample, 10 μg of total RNA was loaded. The probe for *PR-1* was made as described previously by Bowling et al. (*Plant Cell* 9:1573-1584, 1997). As loading controls, the 18S rRNA bands detected by ethidium bromide staining were used.

FIGURE 1C shows the induction of *BGL2-GUS* by different concentrations of INA in wild type (WT), *npr1-1, snil npr1-1*, and *snil NPR1* plants. Ten 2-week-old seedlings grown on MS media containing 0, 0.002, 0.01, and 0.1 mM INA were collected and GUS activity was measured according to the methods described by Jefferson et al. (*EMBO J.* 6:3901-3907, 1987). The values represent the average of 3 replicates ± SE. GUS activity is given as absolute fluorescence units per minute per microgram of protein.

FIGURE 1D shows the morphological phenotypes of wild type (WT), *snil npr1-1,* and *snil NPR1* plants. The plants were grown on soil for 4 weeks before the photograph was taken.

FIGURE 1E shows the growth of Psm ES4326 in wild type (WT), *npr1-1, snil npr1-1*, and *snil NPR1* plants. Four-week-old soil-grown plants were treated with 0.65 mM INA 2 days prior to infection by dipping the plants into a Psm ES4326 suspension (OD600 = 0.2) in 10 mM $MgCl_2$ and 0.01% Silwet L-77 as described by Bowling et al. (*Plant Cell* 6:1845-1857, 1994). Eight leaves were excised for each phenotype, treatment, and time point (0, 1, 2, and 3 days), rinsed with H_2O, weighed, and bacteria were extracted and plated according to methods described by Bowling et al. (*Plant Cell* 6:1845-1857, 1994). Error bars represent 95% confidence limits of log-transformed data from four replicates (Sokal and Rohlf, *Biometry 2nd Edition*, W.H. Freeman and Company, N.Y., 1981). "cfu" represents colony-forming units.

FIGURE 1F shows the average disease rating of *P. parasitica* Noco2 infection in wild type (WT), *npr1-1, snil npr1-1*, and *snil NPR1* plants. Two-week-old seedlings were treated with 0.65 mM INA 2 days prior to infection with a spore suspension (3 x 10^4 spores/ml). The disease symptoms were scored 7 days after the

infection with respect to the number of conidiophores observed on each seedling (30 seedlings were examined for each phenotype and treatment). The scales are defined as follows: 0, no conidiophores on the plants; 1, no more than 5 conidiophores per infected leaf; 2, 6-20 conidiophores on a few infected leaves; 3, 6-20 conidiophores on most infected leaves; 4, 5 or more conidiophores on all infected leaves; 5, 20 or more conidiophores on all infected leaves. Average disease ratings were calculated by summing the scores of all 30 seedlings and dividing by 30. The error bars represent standard errors.

FIGURE 2A is a schematic of the map of the *sni1* locus on chromosome IV The cosmids that complemented the *sni1* mutation are labeled by a "*" and the number of independent transformants tested is shown in parentheses.

FIGURE 2B shows the complementation of *sni1* in *PR* gene expression. Using protocols described in Figure 1, GUS staining was performed on *npr1-1, sni1 npr1-1*, and *sni1 npr1-1* transformed with the complementing cosmid 6-22 while RNA blot analysis was carried out in *sni1 npr1-1* and *sni1 NPR1* transformed with the complementing cosmid 4.

FIGURE 2C shows the complementation of *sni1* in response to pathogen infection. Using the protocols described in Figure 1, *npr1-1, sni1 npr1-1*, and *sni1 npr1* transformed with cosmid 4 were infected by Psm ES4326 and *P. parasitica* Noco2.

FIGURE 3A shows the *SNI1* cDNA sequence (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2). The 11 nucleotides that are deleted in the mRNA as a result of the *sni1* mutation are underlined.

FIGURE 3B shows the *SNI1* genomic DNA sequence (SEQ ID NO:3). Exons are highlighted and start and stop codons are underlined.

FIGURE 3C shows sequence homology between SNI1 and Rb. Using a BLAST search of the GenBank database, SNI1 (SEQ ID NO:4) was found to share 22% amino acid sequence identity and 42% amino acid sequence similarity with the mouse retinoblastoma (Rb) protein (SEQ ID NO:5) in the N domain (Hensey et al., *J. Biol. Chem.* 269:1380-1387, 1994) with an E value of 1.1.

FIGURE 4 shows the nuclear localization of SNI1-GFP. The *35S-SNI1-GFP* DNA preparation (20 μg) was delivered into onion epidermal cells using particle bombardment. After 12 hours of incubation, the GFP fluorescence was observed using a fluorescence microscope with an excitation wavelength of 488 nm. The nuclei in the cells were visible in the bright-field images (data not shown). The *35S-GFP* construct was used as a control.

FIGURE 5 is a schematic showing a model for the regulation of SAR by SNI1 and NPR1. In wild type *(SNI1 NPR1)*, SNI1 is a repressor of *PR* genes (a blocked line) and, therefore, a negative regulator of SAR. Induction of *PR* gene expression (an arrow) and SAR requires both activation of a positive regulator, presumably a TF by SA and derepression (a blocked line) of SNI1 by the SA-activated NPR1. In a *SNI1 npr1-1* mutant, the compromised NPR1 function (dashed oval) results in a failure to derepress SNI1 and a lack of inducible *PR* gene expression and resistance. In a *sni1 npr1* double mutant, the function of SNI1 is compromised (dashed rectangle) and the repression by SNI1 is released even in the absence of an SAR inducer, leading to a background level expression of *PR* genes (a dashed arrow). Activation of the TF is still required for a full-scale induction of *PR* gene expression and SAR. In a *sni1 NPR1* single mutant, the lack of SNI1 function results in a background level of *PR* gene expression.

FIGURE 6 shows the enhanced sensitivity of *sni1* to INA, a chemical inducer of SAR, and the enhanced resistance response to *P. parasitica* Noco infection.

Genetic Dissection of SAR

To further dissect the SAR pathway, a suppressor screen in the *npr1-1* background was conducted. In particular, mutants that restore the inducible *PR* gene expression in the *npr1-1* background have been identified. These mutants most likely harbor mutations in genes that function downstream or parallel to *NPR1* in the SAR pathway. The isolation and characterization of *sni1* (suppressor of *npr1*, inducible 1), a recessive mutant that restores wild type levels of inducible *PR* gene expression and disease resistance is described below. SNI1 represents a new class of SAR regulatory

components. The *SNI1* gene was cloned using a map-based approach and found to encode a novel, leucine-rich, nuclear protein, which shares sequence homology with the animal tumor suppressor retinoblastoma (Rb) (Bernards et al., *Proc. Natl. Acad. Sci. USA* 86:6474-6478, 1989). The sequence data also revealed that the *sni1* mutation causes mis-splicing of the mRNA resulting in a frameshift in the amino terminal region of the protein. Based on the genetic and molecular characterization of *sni1*, a working model is proposed for SNI1 as a negative regulator of SAR.

There now follows a description of the cloning and characterization of a gene encoding a negative regulator of SAR. In addition, a description for isolating additional negative regulators and methods of engineering disease resistant plants is provided. These examples are provided for the purpose of illustrating the invention, and should not be construed as limiting.

Identification of the *sni1* Mutant in the *npr1-1* Background

Seeds of *npr1-1* (40,000), containing the *BGL2-GUS* reporter gene, were mutagenized using 0.3% ethyl-methanesulfonate (EMS) as described by Bowling et al. (*Plant Cell 6*: 1845-1857, 1994), and suppressors of the *npr1-1* mutation were screened in the M_2 population for restored expression of the SAR-responsive *BGL2-GUS* in the presence of the SAR inducer, INA (0.1 mM) as follows. Two-week-old M_2 seedlings grown on Murashige-Skoog (MS) medium (Murashige and Skoog, *Physiol. Plant* 15:473-497, 1962) containing 0.1 mM INA (MS-INA) were tested for GUS activity using 4-methylumbelliferyl β-D-glucuronide (MUG) as substrate (Bowling et al., *Plant Cell* 6:1845-1857, 1994). Those seedlings that showed positive GUS activity were transferred to soil to set seeds. The same screen was then performed on the M_3 progeny. The presence of the *npr1-1* mutation in the suppressor mutants was confirmed by the restriction digestion polymorphism generated by the *npr1-1* mutation. More specifically, PCR primers (F4: 5'GAAGCTATTGGATAGATG3' (SEQ ID NO:6) and R5: 5'GTTGAGCAAGTGCAACT3' (SEQ ID NO:7)) were used to amplify a 770 bp genomic fragment containing the *npr1-1* locus. In *npr1-1*, an NlaIII restriction

digestion site is abolished.

Of 7,000 M_2 plants examined, 13 showed an increase in *BGL2-GUS* expression after induction by INA and were designated *sni* (suppressor of *npr1-1*, inducible) mutants. In particular, the *sni1* mutant exhibited sporadic, weak GUS staining under uninduced conditions but consistent, strong GUS staining after induction by INA (Figure 1A). GUS activity in *sni1* was detected most strongly in the veins of both leaves and roots whereas in wild type, uniform GUS staining was detected in leaves while no GUS staining was seen in roots.

To confirm the effects of *sni* mutations on the expression of endogenous *PR* genes and rule out possible mutants of the *BGL2-GUS* reporter gene, standard RNA blot analysis was performed on all *sni* mutants to identify those which recovered inducible *PR-1* gene expression in the *npr1-1* background. Among these mutants, only *sni1* expresses the *PR-1* gene at levels similar to the wild-type under INA induction (Figure 1B). Consistent with *BGL2-GUS* reporter gene expression (Figure 1A), a higher background expression of *PR-1* was detected in the *sni1* mutant without induction. Because of its interesting phenotypes, *sni1* was chosen for in-depth analyses.

Genetic Characterization of *sni1*

The *sni1 npr1-1* double mutant (BGL2-GUS) was backcrossed with *npr1-1* (*BGL2-GUS*) and the resulting F_1 progeny lost the inducible *BGL2-GUS* expression. These F_1 plants were then allowed to self-fertilize, and the F_2 plants were assayed for the presence or the absence of INA-induced GUS expression using 5-bromo-4-chloro-3-indolyl glucuronide as substrate (Jefferson et al., *EMBO J.* 6:3901-3907, 1987). The obtained segregation ratio for GUS staining was used to determine the Mendelian characteristics of *sni1*. Of 112 F_2 plants examined, 27 had strong GUS staining while the rest had no staining, demonstrating that the *sni1* phenotype is recessive and results from a single nuclear mutation ($\chi^2 = 0.047$; $P>0.5$; Sokal and Rohlf, *Biometry 2nd Edition*, W.H Freeman and Company, New York, 1981). Complementation tests between *sni1* and other recessive *sni* mutants showed

that *sni1* is not allelic to other *sni* mutants. In addition, morphological phenotypes of the F_2 population were found to cosegregate with the *sni1* mutation, namely reduced plant size and pointed first pair of true leaves.

The *sni1 npr1-1* double mutant was also crossed with wild-type plants containing the *BGL2-GUS* reporter gene, and the F_1 progeny lost the background expression of *BGL2-GUS* under uninduced conditions but showed wild-type GUS staining after an INA treatment. The F_2 plants were also examined for segregation of inducible *BGL2-GUS* expression to determine whether the *sni1* phenotype is caused by a reversion of *npr1-1* or by a second-site mutation. If *sni1* is linked to *npr1-1*, all F_2 progeny should have shown inducible *BGL2-GUS* expression. However, of 181 plants tested, 148 showed strong GUS staining while 33 displayed no detectable GUS staining after INA treatment, indicating that *sni1* is not linked to *npr1-1* ($\chi^2 = 0.032$; $P>0.5$; Sokal and Rohlf, *supra*). From the same F_2 population, *sni1 NPR1* was also identified using the reduced-plant-size and the pointed-first-true-leaf phenotypes of *sni1* to detect *sni1* homozygotes and the PCR-NlaIII digestion method to detect *NPR1* homozygotes.

In the wild-type *NPR1* background, the *sni1* mutant (*sni1 NPR1*) expressed *PR-1* and *BGL2-GUS* to a higher level than in *sni1 npr1* under induced conditions (Figures 1B and 1C). As seen in *sni1 npr1* plants, ectopic GUS staining was detected in roots in *sni1 NPR1* plants. In leaves, INA induction resulted in uniform GUS staining. This pattern is different from that observed in the *sni npr1-1* double mutant where GUS staining was detected predominantly in the vascular tissues. This difference in the INA-induced GUS expression between *sni1 npr1-1* and *sni1 NPR1* implied that NPR1 function is still required for high level *PR* gene induction in nonvascular tissues in the *sni1* mutant. Introduction of *sni1* into other *npr1* mutant alleles showed that expression of *sni1* phenotypes is not specific to the *npr1-1* mutant allele (data not shown).

Both *sni1 npr1* and *sni1 NPR1* plants are smaller than wild type (Figure 1D). In *sni1* seedlings, the emerging first pair of true leaves are much narrower than those of the wild type (data not shown). These phenotypes were found to cosegregate with

snil and shown later by complementation analysis to be caused by *snil*. In *snil* mutant plants, no macroscopic or microscopic lesions were detected by trypan blue staining even after an INA treatment (data not shown), indicating that cell death is not involved in causing the *snil* phenotypes.

The Effects of *snil* on SA Accumulation and Sensitivity

The endogenous levels of SA in *snil NPR1* were measured under non-inducing conditions and after infection by an SAR-inducing pathogen *Pseudomonas syringae pv. maculicola* ES4326/*avr*Rpt2 (Psm ES4326/*avr*Rpt2) as follows. Tissues were harvested from 4-week-old, soil-grown plants, ground in liquid N_2, and weighed. SA extraction was performed using a modified protocol derived from Raskin et al. (*Proc. Natl. Acad. Sci. USA* 86:2214-2218, 1989). 3 ml of methanol (90%) was added to the ground tissues and the resulting mixture was vortexed and sonicated for 20 minutes. After centrifugation at 7,000 rpm for 10 minutes the supernatant was collected, the pellet was re-extracted with 2 ml of methanol (100%) and re-centrifuged at 7,000 rpm for another 10 minutes. The supernatants from both extractions were combined and air-dried in a water bath (60°C). The dried samples were resuspended in 2.5 ml 5% trichloroacetic acid (TCA), vortexed, sonicated for 5 minutes, and centrifuged at 7,000 rpm for 10 minutes. The supernatants were collected and extracted in 100/99/1 (vol) ethylacetate/cyclopentane/isopropanol by vigorous vortexing for 10 minutes. The top organic phase was removed and air-dried in a water bath (60°C). The dried extract was resuspended in 250 μl of mobile phase (0.2 M KAc, 0.5 mM EDTA, pH 5), vortexed, and sonicated for 5 minutes, and spun through a 0.22 μm nylon filter (Costar). To measure the amount of extracted SA, the samples were separated through a 100 x 4.6 sperisorb DDS2 column (Keystone Scientific Inc.) with a particle size of 3 μm and a pore size of 80 angstroms at a mobile-phase flow rate of 1 ml/minutes. Fluorescent detection was performed on an HPLC spectrofluorescence detector equipped with a Xenon-mercury arc lamp at an excitation/emission wavelength of 295/405 nm. This procedure had a 25% recovery rate as determined by

extracting known amounts of SA. The results showed that both the base-line and the induced levels of SA found in *sni1* (0.4 ± 0.004 μg/g and 1.3 ± 0.45μg/g, respectively) are comparable to those of the wild type (0.2 ± 0.008 μg/g and 1.7 ± 0.69 μg/g, respectively).

The sensitivity of *sni1 NPR1* and *sni1 npr1-1* mutants to SA and INA was measured by a quantitative assay of the BGL2-GUS reporter gene expression. As shown in Figure 1C, wild type displayed the highest level of *BGL2-GUS* expression at 0.1 mM INA, a concentration normally used for SAR induction while *npr1-1* remained nonresponsive to INA. However, in the *sni1 NPR1* and *sni1 npr1-1* mutants, maximum levels of *BGL2-GUS* expression were reached at a concentration (0.01 mM) ten times lower than in the wild type. Similar results were obtained when SA was used as the inducer (data not shown). Comparing *sni1 NPR1* and *sni1 npr1-1*, higher levels of *BGL2-GUS* were detected for *sni NPR1*, indicating that a functional NPR1 is required for maximum induction of the reporter gene.

Resistance of *sni1* to Pathogens

The effect of *sni1* on pathogen resistance in the *npr1-1* or *NPR1* background was tested using a bacterial pathogen Psm ES4326 which causes leaf spots and an oomycete pathogen *Peronospora parasitica (P. parasitica)* Noco2 which results in downy mildew on uninduced wild type plants as follows.

Infection of wild type and mutant *Arabidopsis* with Psm ES4326 and *P. parasitica* Noco2 was carried out as previously described by Bowling et al. (*Plant Cell* 6:1845-1857, 1994). For the Psm ES4326 infection, 4 samples (8 leaves/sample) were taken for each genotype, treatment, and time point to determine the in planta growth of the bacteria and the 95% confidence limits of the log-transformed data were calculated as described by Sokal and Rohlf (*supra*). For the *P. parasitica* Noco2 infected plants, disease rating was determined for each plant according to Cao et al. (*Proc. Natl. Acad. Sci. USA* 95:6531-6536, 1998). For each genotype and treatment, 30 plants were examined, and an average disease rating was calculated.

After INA induction, wild type plants became resistant to these two pathogens while *npr1-1* remained susceptible (Figures 1E and 1F). In *snil npr1-1*, the *snil* mutation restored the induced resistance. In *snil NPR1*, the effect of *snil* on resistance was insignificant under the infection conditions used, i.e., the pattern of resistance in *snil NPR*1 is indistinguishable from that of the wild type. Although in both *snil npr1-1* and *snil NPR1* background expression of *PR* genes is elevated, these mutants still required further induction to become resistant to Psm ES4326 and *P. parasitica* Noco2 at the inoculants used in these experiments.

Map-Based Cloning of SNI1

The *snil* gene was mapped by crossing *snil npr1-1*, which is in the Columbia ecotype (Col-0, carrying the BGL2-GUS reporter gene), to the *SNI1 NPR1* Landsberg ecotype (La-*er*, also carrying the BGL2-GUS reporter gene). The F_2 progeny homozygous for *snil* were identified in two-week-old plate-grown seedlings using the morphological phenotypes associated with *snil*. The *snil* homozygosity of those progeny critical for determining the map position of *snil* was later confirmed in the F_3 generation using the *snil*-specific *BGL2-GUS* reporter gene expression pattern.

The chromosomal position of *snil* was determined using various CAPS (cleaved amplified polymorphic sequences) markers described by Konieczny and Ausubel (*Plant J.* 4:403-410, 1993). Fifty-eight F_2 progeny homozygous for *snil* were used to determine the crude map position of *snil*. Using CAPS markers SC5 and AG on chromosome IV, 2 and 0 heterozygotes were detected, respectively, indicating that SC5 is ~1.7 cM on the centromeric side of *snil* and AG is closely linked to *snil* (Figure 2A).

For fine mapping, CAPS markers g4539 and g3883-1.4 (http://genome-www.stanford.edu/arabidopsis/aboutcaps.html) were used to examine 718 F_2 progeny homozygous for *snil*. Twenty-three heterozygotes were discovered using g4539 (~1.6 cM on the centromeric side of *snil*) and 22 with g3883-1.4 (~ 1.5 cM on the telomeric side of *snil*). Because the heterozygotes found by these two markers were mutually exclusive, it was determined that *snil* is flanked by these two

markers. No heterozygotes were discovered using the marker ch42 located between g4539 and g3883-1.4, indicating that ch42 is closely linked to *sni1*. Because the ch42 sequence was found in the middle of a bacterial artificial chromosome (BAC) clone, F28J12, which had been completely sequenced by the ESSAII project (http://www.mips.biochem.mpg.de/proj/thal/), two CAPS markers, designated XL3 and XL7, were generated on both sides of ch42 according to the sequence information.

XL3 was amplified using primers 5'CTGGCATCCGTGAAAC3'(SEQ ID NO:8) and 5'GCAGGACTTGATGTATCC3' (SEQ ID NO:9) and the polymorphism between Col and La-*er* was detected by NdeI restriction digestion. XL7 was amplified using primers 5'CCATCCAAAGGCGATAAC3' (SEQ ID NO:10) and 5'CCAAACTACTACCGGATG3' (SEQ ID NO:11) and the polymorphism was revealed by DdeI digestion. These markers were used to narrow down the interval containing *sni1* to a 43.3 kb region through the detection of one mutually exclusive recombinant for each marker, XL7 and XL3.

Because this 43.3 kb region is contained in the BAC clone F28J12, two cosmid libraries were generated from F28J12 using the binary vector pCLD04541 (carrying the kanamycin resistance selection) or pSLJ75516 (carrying the herbicide glufosinate ammonium or "basta" resistance selection). F28J12 was obtained from the Arabidopsis Biological Resource Center at the Ohio State University, and the BAC DNA was prepared according to the protocol provided with the clone. To subclone the BAC, 2 μg of the DNA was partially digested with the TaqI restriction endonuclease for 5 minutes at 60°C, and ligated into the ClaI site of binary vector pCLD04541 or pSLJ75516. The ligated products were then packaged using Gigapack XL (Stratagene) and used to infect E. coli (DH5α).

By probing the libraries with PCR fragments derived from the interval between XL7 and XL3, cosmid contigs spanning the *sni1* region were produced (Figure 2A). These PCR fragments were generated using the following primers: XL4 (F: 5'GTGATGGTGAGGGCTTC3' (SEQ ID NO: 12); R: 5'CGTCGGGATCTACAGG3' (SEQ ID NO:13)), XL7, ch42, XL11 (F:

5'GATGGCAATTGCTGGAG3' (SEQ ID NO:14); R: 5'CTAATGGGATGCGACTC3' (SEQ ID NO:15)) and XL1 (F: 5'GATGAGATGTGCTGAG3' (SEQ ID NO:16); R: 5'CATCGATTTCGCCGTTC3' (SEQ ID NO:17)). The positive clones were then analyzed by restriction digestion using XhoI and HindIII. Because the region was completely sequenced, a contig was easily constructed from both libraries by analyzing the restriction patterns of the clones. The contig made with pCLD04541 was transformed (Clough and Bent, *Plant J.* 16:735-743, 1998) into a *sni1* line (*sni1 npr1-1*) that had lost the *BGL2-GUS* reporter gene and was therefore kanamycin sensitive. The resulting transformants from pCLD04541 clones were selected on MS medium containing 50 mg/ml kanamycin. The contig made with pSLJ75516 was transformed into *sni1 npr1-1* and transformants were selected by spraying ten-day-old soil-grown plants with 0.04% basta (in 0.01% Silwet L-77; Union Carbide, Danbury, CT).

Complementation of *sni1* was determined first by the restoration of wild type morphology and then by the loss of inducible expression of the *BGL2-GUS* reporter gene or the endogenous *PR-1 gene*. Segregation of these phenotypes in the T_2 generation was monitored to distinguish true complementation from contamination by *npr1-1*. PCR was also performed to confirm the presence of the cosmid clone in the complementing transformants using the vector-specific primers.

Complementation of the *sni1* mutation was observed when cosmid clones 4, 7, and 6-22 were transformed into *sni1 npr1-1* mutants. In these transformants, which are *sni1 npr1-1::SNI1*, expression of *PR-1* and the *BGL2-GUS* reporter was completely inhibited, as seen in the *npr1-1* mutant (Figure 2B). The transformants also displayed susceptibility to both Psm ES4326 and *P. parasitica* Noco2 even after an INA induction (Figure 2C). When the cosmids containing *SNI1* was transformed into the *sni1 NPR1* single mutant, the background expression of *PR* genes was repressed; the plants behaved like wild type, showing inducible *PR* gene expression (Figure 2B). The T_2 progeny of the complementing lines all segregated for the *SNI1* and *sni1* phenotypes, which corresponded to the presence and the absence of the selective markers, respectively. This indicated that these are true complementing

transformants rather than contaminants from either *npr1-1* or wild type. Transgenic plants containing cosmids other than clones 4, 7, and 6-22 showed the *sni1* mutant phenotypes.

Sequence Analysis of SNI1

The 15 kb genomic region in the *sni1* mutant corresponding to the insert of the complementing cosmid 4 (Figure 2A) was PCR amplified and sequenced using an ABI automatic sequencer. The *sni1* mutation was identified by aligning the obtained sequence with that of the wild type. In this entire 15 kb region, only one mutation (G to A) was found. The PCR primers used for sequencing were then combined to perform RT-PCR (GeneAmp Kit; Perkin Elmer) and the cDNA sequence in the region flanking the *sni1* locus was determined. The partial cDNA sequence was then used as a probe to screen a cDNA library made in λ ZAPII containing 1-3 kb inserts (Kieber et al., *Cell* 72:427-441, 1993). From the 10^6 plaques screened, one cDNA clone was isolated. The cDNA clone was shown to contain almost full length *SNI1*, missing only 11 nucleotides of the 5' end which was determined by RT-PCR and sequencing. A full length cDNA clone was generated by PCR, put under the control of the constitutive 35S promoter of cauliflower mosaic virus, transformed into *sni1*, and shown to complement the *sni1* mutation. By comparing the genomic and the cDNA sequences, introns were identified in the *SNI1* gene. The G to A mutation in *sni1* was found to be in a 3' intron acceptor site and the effect of this mutation on splicing was detected by RT-PCR of the mutant mRNA and sequencing analysis.

Sequence data were analyzed with different programs available through the internet (http://www.expasy.ch/tools).

Sequence analysis predicted that *SNI1* encodes a novel protein of 432 amino acids (Figure 3A) and molecular weight of 48.8 kDa. Hydropathy plot analysis showed that SNI1 is a soluble protein with no obvious transmembrane domains. A survey of the amino acid sequence showed that SNI1 is a leucine-rich protein (12.7%). A database search for homologs yielded one EST clone isolated from cotton (accession number: AI054954), implying that SNI1 may be conserved in plants. Even

though no substantial homology was found between SNI1 and any known proteins, a short stretch of homology was discovered with the mouse Retinoblastoma (Rb) protein (Bernards et al., *Proc. Natl. Acad. Sci. USA* 86:6474-6478, 1989) (Figure 3C), a tumor suppressor that represses the transcription regulated by transcription factors, such as E2F (Nevins, *Science* 258:424-429, 1992).

Comparison of the cDNA sequence and the genomic sequence revealed that the *SNI1* gene consists of 15 exons (Figure 3B). The G to A mutation in *sni1* occurred in the 3' intron acceptor site at the junction between the fifth intron and the sixth exon. RT-PCR and sequencing analysis of the *sni1* mutant showed that the mutation caused a 11-nucleotide deletion in the cDNA, resulting in a frameshift early in the coding region (Figure 3A, amino acid residue 140).

Expression and Subcellular Localization of SNI1

Standard RNA blot analysis using *Arabidopsis* poly A^+ RNA detected a very faint band of approximately 1500 nucleotides in both uninduced and induced samples (data not shown). In addition, transgenic *Arabidopsis* lines were generated carrying a *SNI1* promoter fusion to the *GUS* reporter gene. Very weak GUS staining was detected mainly in the veins. INA treatment seemed to have no effect on the staining pattern (data not shown).

To determine the subcellular localization of SNI1, the green fluorescent protein (*GFP*) gene was fused to either the 5' or the 3' end of *SNI1* as follows. The *GFP* gene carried by the plasmid pRT2ΔN-mGFP (Stacey et al., *Plant Cell* 11:349-363, 1999) was fused to either the 5' or the 3' end of the *SNI1* gene by inserting the *SNI1* coding sequence into the BglII and NcoI sites of the plasmid, respectively. The resulting plasmids were purified using a Qiagen Midiprep Kit (Valencia, CA), and 20 μg of DNA was bombarded into onion epidermal cells according to a protocol by von Armim and Deng (*Cell* 79: 1035-1045, 1994). GFP was then observed using a fluorescence microscope.

The resulting fusion genes were driven by the constitutive 35S promoter of the cauliflower mosaic virus and the resulting fusion proteins had a MW of 75 kDa, which is above the size exclusion limit (40-60 kDa) for passive diffusion of protein through the nuclear pores (Raikhel, *Plant Physiol.* 100:1627-1632, 1992). When either *35S-GFP-SNI1* or *35S-SNI1-GFP* was delivered into onion epidermal cells by particle bombardment, GFP fluorescence was observed predominantly in the nuclei (Figure 4). Weak fluorescence was also detected in the cytoplasm. When *35S-GFP* was transiently expressed, the smaller GFP protein (26 kDa) was found to be evenly distributed throughout the cells.

Generation of anti-SNI polyclonal antibodies

A polyclonal anti-SNI peptide antibody was raised against the synthetic peptide CFSDPHEGDSISE (SEQ ID NO:18), corresponding to amino acids 381-392 of the SNI polypeptide (Fig. 3A), with the addition of a N-terminal cysteine for later purification. The peptide was synthesized and purified according to standard methods. The purified peptide was next coupled to the carrier molecule, KLH (keyhole limpet hemocyanin). Antibody was obtained by immunizing a white female New Zealand rabbit every 4 weeks with the peptide/KLH conjugate emulsified in Freund's adjuvant according to standard methods. Blood samples were subsequently obtained two weeks after injection of the conjugate and serum was screened by standard western blot analysis. The synthetic peptide without KLH was reduced using Pierce Reduce-Imm Column coupled to a sulfoLink column (Pierce) and was used to purify the antibody according to the manufacturer's instructions. Using standard western blotting techniques, the polyclonal antibody was found to bind specifically to a fragment of SNI1 produced in *E. coli* and plant extracts.

SNI1 as a Negative Regulator of SAR

To identify additional regulators of SAR, a screen for suppressors of *npr1-1* was performed. The recessive mutant *sni1* was further characterized and shown to restore inducible *PR* gene expression and pathogen resistance in the *npr1-1*

background. The *sni1* mutant could only be identified in an *npr1* mutant background because the *sni1 NPR1* single mutant is almost indistinguishable from the wild type with respect to either *PR* gene expression or disease resistance. The recessive, loss-of-function *sni1* represents a new class of SAR-related mutants, distinct from the previously identified, dominant mutants such as *cpr6* and *ssi1* which constitutively express high levels of *PR-1* in the *npr1* background in an SA-dependent fashion (Clarke et al., *Plant Cell* 10:557-569, 1998) or lesion-dependent fashion (Shah et al., *Plant Cell* 11:191-206, 1999).

The characteristics of the *sni1* mutant and the nuclear localization of the SNI1-GFP fusion protein indicate that the wild type SNI1 is a repressor of *PR* gene expression and SAR, which most likely functions downstream of NPR1. The lack of induction of SAR in *npr1* mutants could be explained if a functional, SA-activated NPR1 is required to release the repression of *PR* gene transcription by SNI1. The *sni1* mutation, which causes a frameshift early in the coding region, abolishes SNI1 repression of the *PR* genes and eliminates the requirement for NPR1. Release of SNI1 repression by the mutation also results in elevated background *PR* gene expression in leaves and in roots, and reduced threshold levels of SA and INA that are required to induce *PR* gene expression.

SNI1 appears to represent a novel repressor of transcription, which directly binds to a specific DNA sequence and inhibits the transcriptional machinery. Alternatively, SNI1 may bind to DNA indirectly through interaction with a DNA binding protein because no apparent DNA binding domain has been found in SNI1. In support of this hypothesis, a cis-element, which contains the consensus of a binding site for the plant-specific transcription factor WRKY family, was found in the *PR-1* promoter and shown to negatively regulate the expression of the gene (Lebel et al., *Plant J.* 16:223-233, 1998). Mutations in this element result in an expression pattern of *PR-1* similar to that observed in the *sni1* mutant, i.e., an elevated background expression and an enhanced response to INA induction. It is equally plausible that SNI1 affects transcription by sequestering a transcriptional activator. The stretch of sequence homology detected between SNI1 and the tumor suppressor gene *Rb*

suggests such a possibility because Rb negatively regulates gene expression by interacting with transcription factors such as E2F (Nevins, *Science* 258:424-429, 1992). The biological function of the homologous region in Rb, known as the N domain (Hensey et al., *J. Biol. Chem.* 269:1380-1387, 1994), is still unclear even though it is highly conserved among Rb homologs isolated from many organisms. Cell culture and *in vitro* experiments have indicated that this domain is dispensable for Rb function (Jacks et al., *Nature* 359:295-300, 1992; Lee et al., *Nature* 359:288-294, 1992, *Genes Dev.* 8:2008-2021 1994; Fung et al., *Oncogene* 8:2659-2672, 1993). However, deletion mutants of this domain failed to rescue Rb mutant mice, suggesting that this domain may in fact be of functional importance (Riley et al., *Mol. Cell. Biol.* 17:7342-7352, 1997).

The inducible, rather than constitutive, *PR* gene expression and disease resistance observed in *sni1 npr1-1* suggests the requirement of a separate activation event in inducing *PR* gene expression and SAR in addition to the regulations of NPR1 and SNI1. This parallel activation event only became evident when both the NPR1 and SNI1 functions were abolished. SA is required for this induction event because expression of the SA-degrading salicylate hydroxylase gene (*nahG*) in the *sni1 npr1* mutant prevents the induction of the *PR* genes by exogenous application of SA (data not shown). This activation event probably does not require the function of NPR1 but may be facilitated by NPR1 because the *PR* gene induction that occurs in *sni1 npr1-1* is less dramatic than in *sni1 NPR1*. Indeed, in a yeast two-hybrid screen, NPR1 was found to bind specifically to a subclass of bZIP transcription factors. These bZIP transcription factors may be the positive regulators of the *PR-1* gene that are activated by SA, in parallel to NPR1, to induce *PR* gene expression and SAR. In support of this, mutations in the binding site for the bZIP transcription factors in the *PR-1* promoter abolish the inducibility of *PR-1* by SA and INA (Lebel et al., *Plant J.* 16:223-233, 1998). The consequence of the NPR1-bZIP transcription factor interaction has yet to be revealed. Binding of NPR1 to the bZIP transcription factors may facilitate the activation of these transcription factors or recruit NPR1 to its functional location.

Taking all the data together, a working model to explain the signal transduction pathway leading to the activation of *PR* gene expression and illustrate the roles of SNI1 and NPR1 in the induction of SAR is shown in Figure 5. In wild type plants, induction of *PR* genes and SAR by SA or INA may require two separate events: inactivation of SNI1 by SA through a function of NPR1 and activation of a positive regulator, presumably the bZIP transcription factor, by SA through a mechanism that does not require NPR1 but may be facilitated by NPR1. In an *npr1* mutant, where SNI1 repression cannot be removed due to the lack of NPR1 activity, induction of SAR is blocked. However, in the *sni1 npr1-1* double mutant, where *PR* gene expression is no longer repressed due to the *sni1* mutation, the requirement for NPR1 is eliminated and background levels of *PR* gene expression are observed under uninduced conditions. The lack of SNI1 repression may also explain the elevated basal expression of *PR* genes in the *sni1 NPR1* single mutant. When a wild type *SNI1* gene was transformed into *sni1 NPR1* and *sni1 npr1-1,* this background expression of *PR* genes was repressed and the phenotype of the transformants reverted back to wild type and *npr1-1*, respectively.

Identification and cloning of *SNI1* provides a basis to further unravel the regulatory mechanism of *PR* gene expression and SAR. The data described herein suggests that induction of SAR involves both activation and derepression events. The significance of SNI1 repression is underlined by the leakiness in *PR* gene regulation observed in *sni1* mutants under noninducing conditions and by the reduced plant size of these mutants. Identification of *sni1* also suggests a possible means of engineering plants with enhanced disease resistance. In particular, disease resistance analysis has shown that disruption of SNI1 function resulted in enhanced sensitivity to chemical inducers of SAR such as SA and INA, as well as resulting in an enhanced resistance response to pathogens such as *Peronospora parasitica* (Figure 6).

Microarray Gene Expression Monitoring and Discovery

The cells, plants and plant components, and methods described herein may also be used in combination with standard microarray technologies (for example,

cDNA microarrays or DNA chips) for monitoring the expression of genes controlled by or associated with negative regulators of SAR. In particular, microarray expression monitoring provides a basis for understanding how SNI1 integrates into the SAR signal transduction pathway. Moreover, such technology enables the identification of novel genes associated with the SAR pathway. For example, target genes that regulate or are regulated by SNI1 may be identified by correlating changes in gene expression with specific changes in cells having either reduced or increased levels of the SAR negative regulator protein. Genes exhibiting altered expression levels in such cells are identified and subsequently serve as specific targets whose expression is modified to generate transgenic plants having increased resistance to a pathogen.

In general, microarray technology utilizes organized DNA libraries of interest (for example, a cDNA library) whose members are arrayed on a solid support (e.g., a glass slide). Such cDNAs are amplified by polymerase chain reaction, purified, and small quantities are deposited onto known locations of a microchip according to standard methods such as high-speed robotics. Microarrays of cDNAs accordingly provide expression information for each gene represented on the microchip. Arrayed sequences, in turn, serve as targets for hybridization to cDNA probes prepared from RNA samples of plant cells or tissues of interest. Multi-color fluorescence labeling of cDNA probes further provides a means facilitating comparative differential expression analysis of a variety of plant tissues under different physiological and environmental conditions. Typically, a gene whose expression is increased or decreased in an appropriate genetic background is considered as a useful target involved in plant disease resistance.

Exemplary microarray methods for differential and quantitative monitoring of gene expression and for identifying novel genes are provided in Schena et al. (*Science* 270:467-470, 1995), Schena et al. (*Proc. Natl. Acad. Sci. USA* 93:10614-10619, 1996), Heuller et al. (*Proc. Natl. Acad. Sci. USA* 94:2150-2155, 1997), and Case-Green et al. (*Curr. Opin. Chem. Biol.* 2:404-410, 1998).

In one particular example, microarrays may be used to examine gene expression in plant tissue (e.g., a leaf or root) having reduced levels of a negative regulator of SAR. For example, plants having reduced SNI1 levels (such as plants expressing a dominant-negative SNI1 or that express antisense SNI1 RNA) are resistant to pathogens, whereas in wild type plants, SNI1 is a repressor of *PR* genes, and, therefore, a negative regulator of SAR. Total mRNA from leaves of SA-induced wild-type *Arabidopsis* (control) and a *sni* mutant test plant is fluorescently labeled using a single round of reverse transcriptase. Fluorescently labeled mRNA is then hybridized to a microarray containing target DNA, such as expressed sequence tags from an *Arabidopsis* cDNA library. The array is washed under high stringency conditions and scanned with a confocal laser scanning device to detect hybridization via emission of the fluorescently labeled DNA. Comparative expression analysis of control and test plants are determined, and array elements displaying altered fluorescence ratios are selected for further analysis. For example, genes highly induced in the test plants as compared to wild-type plants are selected and characterized by sequencing. Such genes may then be used to engineer disease resistant plants.

Alternatively, microarrays may be used to examine gene expression in plant tissue having increased levels of a negative regulator of SAR (for example, plants overexpressing wild-type SNI1 or plants expressing wild-type SNI1 protein in a *sni1 npr1* genetic background). In such plants, SAR is repressed, abolishing induced *PR* gene expression and resistance. Accordingly, these plants have increased susceptibility to their pathogens and provide a means for identifying novel target genes that regulate negative regulators of SAR such as SNI1.

In one working example, total mRNA from roots of INA-induced wild-type *Arabidopsis* (control) and test plants expressing wild-type SNI1 in an *sni1 npr1* background is fluorescently labeled and hybridized to a microarray as described above. The array is washed and analyzed using a confocal laser scanning device. Comparative expression data between control and test plants is determined, and array elements displaying altered fluorescence ratios are then selected for further analysis.

For example, genes having decreased expression in the test plants as compared to wild type are then selected and characterized by sequencing.

Isolation of Other Genes Encoding Negative Regulator Polypeptides of SAR

Any plant cell can serve as the nucleic acid source for the molecular cloning of a gene encoding a negative regulator polypeptide of SAR. Isolation of such a negative regulator gene involves the isolation of those DNA sequences which encode a protein exhibiting negative regulator polypeptide of SAR-associated structures, properties, or activities, for example, those described herein for *SNI1*. Based on the negative regulator genes and polypeptides described herein, the isolation of additional sequences coding for negative regulators of SAR is made possible using standard strategies and techniques that are well known in the art.

In one particular example, the negative regulator sequences described herein (for example, those described for *SNI1*) may be used, together with conventional screening methods of nucleic acid hybridization screening. Such hybridization techniques and screening procedures are well known to those skilled in the art and are described, for example, in Benton and Davis, *Science* 196:180-182, 1977; Grunstein and Hogness, *Proc. Natl. Acad. Sci., USA* 72:3961-3695, 1975; Ausubel et al., 1999, *Current Protocols in Molecular Biology*, Wiley Interscience, New York; Berger and Kimmel (*supra*); and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York. In one particular example, all or part of the *SNI1* cDNA (described herein) may be used as a probe to screen a recombinant plant DNA library for genes having sequence identity to a gene encoding a negative regulator polypeptide of SAR. Hybridizing sequences are detected by plaque or colony hybridization according to the methods described below.

Alternatively, using all or a portion of the amino acid sequence of a negative regulator polypeptide of SAR (for example, SNI1), one may readily design specific oligonucleotide probes, including degenerate oligonucleotide probes (i.e., a mixture of all possible coding sequences for a given amino acid sequence). These oligonucleotides may be based upon the sequence of either DNA strand and any

appropriate portion of the negative regulator polypeptide gene sequence. General methods for designing and preparing such probes are provided, for example, in Ausubel et al. (*supra*), and Berger and Kimmel, *Guide to Molecular Cloning Techniques*, 1987, Academic Press, New York. These oligonucleotides are useful for gene isolation, either through their use as probes capable of hybridizing to complementary sequences or as primers for various amplification techniques, for example, polymerase chain reaction (PCR) cloning strategies. If desired, a combination of different oligonucleotide probes may be used for the screening of a recombinant DNA library. The oligonucleotides may be detectably-labeled using methods known in the art and used to probe filter replicas from a recombinant DNA library. Recombinant DNA libraries are prepared according to methods well known in the art, for example, as described in Ausubel et al. (*supra*), or they may be obtained from commercial sources.

In one particular example of this approach, gene sequences having greater than 80% identity to *SNI1* are detected or isolated using high stringency conditions. High stringency conditions may include hybridization at about 42°C and about 50% formamide, 0.1 mg/ml sheared salmon sperm DNA, 1% SDS, 2X SSC, 10% Dextran sulfate, a first wash at about 65°C, about 2X SSC, and 1% SDS, followed by a second wash at about 65°C and about 0.1X SSC. Alternatively, high stringency conditions may include hybridization at about 42°C and about 50% formamide, 0.1 mg/ml sheared salmon sperm DNA, 0.5% SDS, 5X SSPE, 1X Denhardt's, followed by two washes at room temperature and 2X SSC, 0.1% SDS, and two washes at between 55-60°C and 0.2X SSC, 0.1% SDS.

In another approach, low stringency hybridization conditions for detecting negative regulator genes having about 30% or greater sequence identity to *SNI1* described herein include, for example, hybridization at about 42°C and 0.1 mg/ml sheared salmon sperm DNA, 1% SDS, 2X SSC, and 10% Dextran sulfate (in the absence of formamide), and a wash at about 37°C and 6X SSC, about 1% SDS. Alternatively, the low stringency hybridization may be carried out at about 42°C and 40% formamide, 0.1 mg/ml sheared salmon sperm DNA, 0.5% SDS, 5X SSPE, 1X

Denhardt's, followed by two washes at room temperature and 2X SSC, 0.1% SDS and two washes at room temperature and 0.5X SSC, 0.1% SDS. These stringency conditions are exemplary; other appropriate conditions may be determined by those skilled in the art.

As discussed above, oligonucleotides may also be used as primers in amplification cloning strategies, for example, using PCR. PCR methods are well known in the art and are described, for example, in *PCR Technology*, Erlich, ed., Stockton Press, London, 1989; *PCR Protocols: A Guide to Methods and Applications*, Innis et al., eds., Academic Press, Inc., New York, 1990; and Ausubel et al. (*supra*). Primers encoding specific structural features of a negative regulator polypeptide of SAR, for example, the N domain motif, are useful for isolating genes having similar structural domains. Primers are optionally designed to allow cloning of the amplified product into a suitable vector, for example, by including appropriate restriction sites at the 5' and 3' ends of the amplified fragment (as described herein). If desired, negative regulator gene sequences may be isolated using the PCR "RACE" technique, or Rapid Amplification of cDNA Ends (see, for example, Innis et al. (*supra*)). By this method, oligonucleotide primers based on an negative regulator gene sequence (for example, *SNI1*) are oriented in the 3' and 5' directions and are used to generate overlapping PCR fragments. These overlapping 3'- and 5'-end RACE products are combined to produce an intact full-length cDNA. This method is described in Innis et al. (*supra*); and Frohman et al., *Proc. Natl. Acad. Sci. USA* 85:8998- 9002, 1988.

Alternatively, any plant cDNA or cDNA expression library may be screened by functional complementation of a plant having a mutation in a gene encoding a negative regulator polypeptide of SAR (for example, a *sni1* mutant) according to standard methods described herein.

Confirmation of a sequence's relatedness to the negative regulator polypeptide of SAR gene family may be accomplished by a variety of conventional methods including, but not limited to, functional complementation assays and sequence comparison of the gene and its expressed product. In addition, the activity of the gene product may be evaluated according to any of the techniques described herein, for

example, the functional or immunological properties of its encoded product.

In addition, the *SNI1* sequence disclosed herein provides a basis for searching databases such as Genbank to identify *SNI1* homologs.

Once a gene encoding a negative regulator polypeptide of SAR is identified, it is cloned according to standard methods and used for the construction of plant expression vectors as described below.

Polypeptide Expression

Polypeptides of the invention may be expressed and produced by transformation of a suitable host cell with all or part of a gene encoding a negative regulator polypeptide of SAR (for example, the *SNI1* cDNA described herein) in a suitable expression vehicle or with a plasmid construct engineered for increasing the expression of the encoded polypeptide *in vivo*.

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems may be used to provide the recombinant protein. The precise host cell used is not critical to the invention. The polypeptide may be produced in a prokaryotic host, for example, *E. coli*, or in a eukaryotic host, for example, *Saccharomyces cerevisiae*, mammalian cells (for example, COS 1 or NIH 3T3 cells), or any of a number of plant cells or whole plant including, without limitation, algae, tree species, ornamental species, temperate fruit species, tropical fruit species, vegetable species, legume species, crucifer species, monocots, dicots, or in any plant of commercial or agricultural significance. Particular examples of suitable plant hosts include, but are not limited to, conifers, petunia, tomato, potato, pepper, tobacco, *Arabidopsis*, lettuce, sunflower, oilseed rape, flax, cotton, grape, citrus, sugarbeet, celery, soybean, alfalfa, *Medicago*, lotus, *Vigna*, cucumber, carrot, eggplant, cauliflower, horseradish, morning glory, poplar, walnut, apple, grape, asparagus, cassava, rice, maize, millet, onion, barley, orchard grass, oat, rye, and wheat.

Such cells are available from a wide range of sources including the American Type Culture Collection (Rockland, MD); or from any of a number seed companies, for example, W. Atlee Burpee Seed Co. (Warminster, PA), Park Seed Co. (Greenwood, SC), Johnny Seed Co. (Albion, ME), or Northrup King Seeds (Harstville, SC). Descriptions and sources of useful host cells are also found in Vasil I.K., *Cell Culture and Somatic Cell Genetics of Plants*, Vol I, II, III Laboratory Procedures and Their Applications Academic Press, New York, 1984; Dixon, R.A., *Plant Cell Culture-A Practical Approach*, IRL Press, Oxford University, 1985; Green et al., *Plant Tissue and Cell Culture*, Academic Press, New York, 1987.

For prokaryotic expression, DNA encoding a negative regulator polypeptide of SAR is carried on a vector operably linked to control signals capable of effecting expression in the prokaryotic host. If desired, the coding sequence may contain, at its 5' end, a sequence encoding any of the known signal sequences capable of effecting secretion of the expressed protein into the periplasmic space of the host cell, thereby facilitating recovery of the protein and subsequent purification. Prokaryotes most frequently used are various strains of *E. coli*; however, other microbial strains may also be used. Plasmid vectors are used which contain replication origins, selectable markers, and control sequences derived from a species compatible with the microbial host. Examples of such vectors are found in Pouwels et al. (*supra*) or Ausubel et al. (*supra*). Commonly used prokaryotic control sequences (also referred to as "regulatory elements") are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences. Promoters commonly used to direct protein expression include the beta-lactamase (penicillinase), the lactose (lac) (Chang et al., *Nature* 198:1056, 1977), the tryptophan (Trp) (Goeddel et al., *Nucl. Acids Res.* 8:4057-4074, 1980), and the *tac* promoter systems, as well as the lambda-derived P_L promoter and N-gene ribosome binding site (Simatake et al., *Nature* 292:128-132, 1981).

One particular bacterial expression system for polypeptide production is the *E. coli* pET expression system (Novagen, Inc., Madison, WI). According to this expression system, DNA encoding a negative regulator polypeptide (for example,

SNI1) is inserted into a pET vector in an orientation designed to allow expression. Since the negative regulator gene is under the control of the T7 regulatory signals, expression of the gene is induced by inducing the expression of T7 RNA polymerase in the host cell. This is typically achieved using host strains which express T7 RNA polymerase in response to IPTG induction. Once produced, the recombinant negative regulator polypeptide is then isolated according to standard methods known in the art, for example.

Another bacterial expression system for polypeptide production is the pGEX expression system (Pharmacia). This system employs a GST gene fusion system which is designed for high-level expression of genes or gene fragments as fusion proteins with rapid purification and recovery of functional gene products. The protein of interest is fused to the carboxyl terminus of the glutathione S-transferase protein from *Schistosoma japonicum* and is readily purified from bacterial lysates by affinity chromatography using Glutathione Sepharose 4B. Fusion proteins can be recovered under mild conditions by elution with glutathione. Cleavage of the glutathione S-transferase domain from the fusion protein is facilitated by the presence of recognition sites for site-specific proteases upstream of this domain. For example, proteins expressed in pGEX-2T plasmids may be cleaved with thrombin; those expressed in pGEX-3X may be cleaved with factor Xa.

For eukaryotic expression, the method of transformation or transfection and the choice of vehicle for expression of the negative regulator polypeptide of SAR will depend on the host system selected. Transformation and transfection methods are described, for example, in Ausubel et al. (*supra*); Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989; Gelvin et al., *Plant Molecular Biology Manual*, Kluwer Academic Publishers, 1990; Kindle, K., *Proc. Natl. Acad. Sci. USA* 87:1228, 1990; Potrykus, I., *Gene Transfer to Plants: A Laboratory Manual*, Springer-Verlag, 1995; and BioRad (Hercules, CA) Technical Bulletin #1687 (Biolistic Particle Delivery Systems). Expression vehicles may be chosen from those provided, for example, in *Cloning Vectors: A Laboratory Manual* (P.H. Pouwels et al., 1985, Supp. 1987); Clontech Molecular Biology Catalog (Catalog 1992/93 Tools

for the Molecular Biologist, Palo Alto, CA); and the references cited above. Other expression constructs are described by Fraley et al. (U.S. Pat. No. 5,352,605).

Construction of Plant Transgenes

Most preferably, a negative regulator polypeptide of SAR (for example, SNI1) is produced by a stably-transfected plant cell line, a transiently-transfected plant cell line, or by a transgenic plant. A number of vectors suitable for stable or extrachromosomal transfection of plant cells or for the establishment of transgenic plants are available to the public; such vectors are described in Pouwels et al. (*supra*), Weissbach and Weissbach (*supra*), and Gelvin et al. (*supra*). Methods for constructing such cell lines are described in, for example, Weissbach and Weissbach (*supra*), and Gelvin et al. (*supra*).

Typically, plant expression vectors include (1) a cloned plant gene under the transcriptional control of 5' and 3' regulatory sequences and (2) a dominant selectable marker. Such plant expression vectors may also contain, if desired, a promoter regulatory region (for example, one conferring inducible or constitutive, pathogen- or wound-induced, environmentally- or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

Once the desired nucleic acid sequence is obtained as described above, it may be manipulated in a variety of ways known in the art. For example, where the sequence involves non-coding flanking regions, the flanking regions may, if desired, be subjected to mutagenesis or deleted.

The negative regulator of SAR sequence may, if desired, be combined with other DNA sequences in a variety of ways. For example, the DNA sequence of the invention may be employed with all or part of the gene sequences normally associated with the expression of a negative regulator polypeptide. In its component parts, a DNA sequence encoding a negative regulator polypeptide of SAR is combined in a DNA construct having a transcription initiation control region capable of promoting

transcription and translation in a host cell.

In general, the constructs will involve regulatory regions functional in plants which provide for modified production of a negative regulator polypeptide of SAR as discussed herein. The open reading frame coding for the negative regulator polypeptide of SAR or functional fragment thereof will be joined at its 5' end to a transcription initiation regulatory region such as the sequence naturally found in the 5' upstream region of the negative regulator structural gene. Numerous other transcription initiation regions are available which provide for constitutive or inducible regulation.

For applications where developmental, cell, tissue, hormonal, or environmental expression is desired, appropriate 5' upstream non-coding regions are obtained from other genes, for example, from genes regulated during meristem development, seed development, embryo development, or leaf development.

Regulatory transcript termination regions may also be provided in DNA constructs of this invention as well. Transcript termination regions may be provided by the DNA sequence encoding the negative regulator polypeptide of SAR or any convenient transcription termination region derived from a different gene source. The transcript termination region will contain preferably at least 1-3 kb of sequence 3' to the structural gene from which the termination region is derived. Plant expression constructs having a negative regulator polypeptide of SAR as the DNA sequence of interest for expression (in either the sense or antisense orientation) may be employed with a wide variety of plant life. Such genetically-engineered plants are useful for a variety of industrial and agricultural applications as discussed infra, including the generation of disease resistant plants. Importantly, this invention is applicable to dicotyledons and monocotyledons, and will be readily applicable to any new or improved transformation or regeneration method.

The expression constructs include at least one promoter operably linked to at least one negative regulator polypeptide of SAR gene. An example of a useful plant promoter according to the invention is a caulimovirus promoter, for example, a cauliflower mosaic virus (CaMV) promoter. These promoters confer high levels of

expression in most plant tissues, and the activity of these promoters is not dependent on virally encoded proteins. CaMV is a source for both the 35S and 19S promoters. Examples of plant expression constructs using these promoters are found in Fraley et al., U.S. Pat. No. 5,352,605. In most tissues of transgenic plants, the CaMV 35S promoter is a strong promoter (see, for example, Odell et al., *Nature* 313:810-812, 1985). The CaMV promoter is also highly active in monocots (see, for example, Dekeyser et al., *Plant Cell* 2:591-602, 1990). Moreover, activity of this promoter can be further increased (i.e., between 2-10 fold) by duplication of the CaMV 35S promoter (see for example, Fang et al., *Plant Cell* 1:141-150, 1989 or McPherson and Kay, U.S. Pat. No. 5,378,142).

Other useful plant promoters include, without limitation, the nopaline synthase (NOS) promoter (Rogers and Fraley, U.S. Pat. No. 5,034,322), the octopine synthase promoter (Fromm et al., *Plant Cell* 1:977-984, 1989), figwort mosiac virus (FMV) promoter (Rogers, U.S. Pat. No. 5,378,619), the rice actin promoter (Wu and McElroy, WO 91/09948), and the ubiquitin promoter system (Quail et al., U.S. Pat. 5,614,399).

Exemplary monocot promoters include, without limitation, commelina yellow mottle virus promoter, sugar cane badna virus promoter, rice tungro bacilliform virus promoter, maize streak virus element, and wheat dwarf virus promoter.

For certain applications, it may be desirable to produce the negative regulator polypeptide of SAR in an appropriate tissue, at an appropriate level, or at an appropriate developmental time. For this purpose, there are an assortment of gene promoters, each with its own distinct characteristics embodied in its regulatory sequences, shown to be regulated in response to inducible signals such as the environment, hormones, and/or developmental cues. These include, without limitation, gene promoters that are responsible for heat-regulated gene expression, light-regulated gene expression (for example, the pea *rbcS-3A*; the maize *rbcS* promoter; the chlorophyll a/b-binding protein gene found in pea; or the Arabssu promoter), hormone-regulated gene expression (for example, the abscisic acid (ABA) responsive sequences from the *Em* gene of wheat; the ABA-inducible HVA1 and

HVA22, and rd29A promoters of barley and *Arabidopsis*; and wound-induced gene expression (for example, of *wunI*), organ-specific gene expression (for example, of the tuber-specific storage protein gene; the 23-kDa zein gene from maize described by; or the French bean β-phaseolin gene), or pathogen-inducible promoters (for example, the *PR-1*, *prp-1*, or β-1,3 glucanase promoters, the fungal-inducible *wirla* promoter of wheat, and the nematode-inducible promoters, *TobRB7-5A* and *Hmg-1*, of tobacco and parsley, respectively).

Plant expression vectors may also optionally include RNA processing signals, e.g, introns, which have been shown to be important for efficient RNA synthesis and accumulation (Callis et al., *Genes and Dev.* 1:1183-1200, 1987). The location of the RNA splice sequences can dramatically influence the level of transgene expression in plants. In view of this fact, an intron may be positioned upstream or downstream of a negative regulator polypeptide of SAR-encoding sequence in the transgene to modulate levels of gene expression.

In addition to the aforementioned 5' regulatory control sequences, the expression vectors may also include regulatory control regions which are generally present in the 3' regions of plant genes. For example, the 3' terminator region may be included in the expression vector to increase stability of the mRNA. One such terminator region may be derived from the PI-II terminator region of potato. In addition, other commonly used terminators are derived from the octopine or nopaline synthase signals.

The plant expression vector also typically contains a dominant selectable marker gene used to identify those cells that have become transformed. Useful selectable genes for plant systems include genes encoding antibiotic resistance genes, for example, those encoding resistance to hygromycin, kanamycin, bleomycin, G418, streptomycin, or spectinomycin. Genes required for photosynthesis may also be used as selectable markers in photosynthetic-deficient strains. Finally, genes encoding herbicide resistance may be used as selectable markers; useful herbicide resistance genes include the *bar* gene encoding the enzyme phosphinothricin acetyltransferase and conferring resistance to the broad spectrum herbicide Basta® (Hoechst Marion

Rousel, Frankfurt, Germany).

Efficient use of selectable markers is facilitated by a determination of the susceptibility of a plant cell to a particular selectable agent and a determination of the concentration of this agent which effectively kills most, if not all, of the transformed cells. Some useful concentrations of antibiotics for tobacco transformation include, for example, 20-100 μg/ml (kanamycin), 20-50 μg/ml (hygromycin), or 5-10 μg/ml (bleomycin). A useful strategy for selection of transformants for herbicide resistance is described, for example, by Vasil et al., *supra*.

In addition, if desired, the plant expression construct may contain a modified or fully-synthetic structural negative regulator polypeptide of SAR coding sequence which has been changed to enhance the performance of the gene in plants. Methods for constructing such a modified or synthetic gene are described in Fischoff and Perlak, U.S. Pat. No. 5,500,365.

It should be readily apparent to one skilled in the art of molecular biology, especially in the field of plant molecular biology, that the level of gene expression is dependent, not only on the combination of promoters, RNA processing signals, and terminator elements, but also on how these elements are used to increase the levels of selectable marker gene expression.

Plant Transformation

Upon construction of the plant expression vector, several standard methods are available for introduction of the vector into a plant host, thereby generating a transgenic plant. These methods include (1) *Agrobacterium*-mediated transformation (*A. tumefaciens* or *A. rhizogenes*) (see, for example, Lichtenstein and Fuller In: *Genetic Engineering*, vol 6, PWJ Rigby, ed, London, Academic Press, 1987; and Lichtenstein, C.P., and Draper, J,. In: *DNA Cloning*, Vol II, D.M. Glover, ed, Oxford, IRI Press, 1985)), (2) the particle delivery system, (3) microinjection protocols, (4) polyethylene glycol (PEG) procedures, (5) liposome-mediated DNA uptake, (6) electroporation protocols, (7) the vortexing method, or (8) the so-called whiskers methodology (see, for example, Coffee et al., U.S. Pat. 5,302,523). The method of

transformation is not critical to the invention. Any method which provides for efficient transformation may be employed. As newer methods are available to transform crops or other host cells, they may be directly applied. Suitable plants for use in the practice of the invention include, but are not limited to, sugar cane, wheat, rice, maize, sugar beet, potato, barley, manioc, sweet potato, soybean, sorghum, cassava, banana, grape, oats, tomato, millet, coconut, orange, rye, cabbage, apple, watermelon, canola, cotton, carrot, garlic, onion, pepper, strawberry, yam, peanut, onion, bean, pea, mango, citrus plants, walnuts, and sunflower.

The following is an example outlining one particular technique, an *Agrobacterium*-mediated plant transformation. By this technique, the general process for manipulating genes to be transferred into the genome of plant cells is carried out in two phases. First, cloning and DNA modification steps are carried out in *E. coli*, and the plasmid containing the gene construct of interest is transferred by conjugation or electroporation into *Agrobacterium*. Second, the resulting *Agrobacterium* strain is used to transform plant cells. Thus, for the generalized plant expression vector, the plasmid contains an origin of replication that allows it to replicate in *Agrobacterium* and a high copy number origin of replication functional in *E. coli*. This permits facile production and testing of transgenes in *E. coli* prior to transfer to *Agrobacterium* for subsequent introduction into plants. Resistance genes can be carried on the vector, one for selection in bacteria, for example, streptomycin, and another that will function in plants, for example, a gene encoding kanamycin resistance or herbicide resistance. Also present on the vector are restriction endonuclease sites for the addition of one or more transgenes and directional T-DNA border sequences which, when recognized by the transfer functions of *Agrobacterium*, delimit the DNA region that will be transferred to the plant.

In another example, plant cells may be transformed by shooting into the cell tungsten microprojectiles on which cloned DNA is precipitated. In the Biolistic Apparatus (Bio-Rad) used for the shooting, a gunpowder charge (22 caliber Power Piston Tool Charge) or an air-driven blast drives a plastic macroprojectile through a gun barrel. An aliquot of a suspension of tungsten particles on which DNA has been

precipitated is placed on the front of the plastic macroprojectile. The latter is fired at an acrylic stopping plate that has a hole through it that is too small for the macroprojectile to pass through. As a result, the plastic macroprojectile smashes against the stopping plate, and the tungsten microprojectiles continue toward their target through the hole in the plate. For the instant invention the target can be any plant cell, tissue, seed, or embryo. The DNA introduced into the cell on the microprojectiles becomes integrated into either the nucleus or the chloroplast.

In general, transfer and expression of transgenes in plant cells are now routine practices to those skilled in the art, and have become major tools to carry out gene expression studies in plants and to produce improved plant varieties of agricultural or commercial interest.

Transgenic Plant Regeneration

Plant cells transformed with a plant expression vector can be regenerated, for example, from single cells, callus tissue, or leaf discs according to standard plant tissue culture techniques. It is well known in the art that various cells, tissues, and organs from almost any plant can be successfully cultured to regenerate an entire plant; such techniques are described, for example, in Vasil *supra*; Green et al., *supra*; Weissbach and Weissbach, *supra*; and Gelvin et al., *supra*.

In one particular example, an expression construct containing a cloned negative regulator polypeptide of SAR under the control of the 35S CaMV promoter and the nopaline synthase terminator and carrying a selectable marker (for example, kanamycin resistance) is transformed into *Agrobacterium*. Transformation of leaf discs (for example, of tobacco or potato leaf discs) with vector-containing *Agrobacterium* is carried out as described by Horsch et al. (*Science* 227:1229-1231, 1985). Putative transformants are selected after a few weeks (for example, 3 to 5 weeks) on plant tissue culture media containing kanamycin (for example 100 μg/ml). Kanamycin-resistant shoots are then placed on plant tissue culture media without hormones for root initiation. Kanamycin-resistant plants are then selected for greenhouse growth. If desired, seeds from self-fertilized transgenic plants can then be

sowed in a soil-less medium and grown in a greenhouse. Kanamycin-resistant progeny are selected by sowing surfaced sterilized seeds on hormone-free kanamycin-containing media. Analysis for the integration of the transgene is accomplished by standard techniques (see, for example, Ausubel et al. *supra*; Gelvin et al. *supra*).

Transgenic plants expressing the selectable marker are then screened for transmission of the transgene DNA by standard immunoblot and DNA detection techniques. Each positive transgenic plant and its transgenic progeny are unique in comparison to other transgenic plants established with the same transgene. Integration of the transgene DNA into the plant genomic DNA is in most cases random, and the site of integration can affect the levels and the tissue and developmental patterns of transgene expression. Consequently, a number of transgenic lines are usually screened for each transgene to identify and select plants with the most appropriate expression profiles.

Transgenic lines are evaluated for levels of transgene expression. Expression at the RNA level is determined initially to identify and quantitate expression-positive plants. Standard techniques for RNA analysis are employed and include PCR amplification assays using oligonucleotide primers designed to amplify only transgene RNA templates and solution hybridization assays using transgene-specific probes (see, for example, Ausubel et al., *supra*). The RNA-positive plants are then analyzed for protein expression by western immunoblot analysis using negative regulator polypeptide specific antibodies (see, for example, Ausubel et al., *supra*). In addition, *in situ* hybridization and immunocytochemistry according to standard protocols can be done using transgene-specific nucleotide probes and antibodies, respectively, to localize sites of expression within transgenic tissue.

In addition, if desired, once the recombinant negative polypeptide is expressed in any cell or in a transgenic plant (for example, as described above), it may be isolated, for example, using affinity chromatography. In one example, an anti-SNI1 polypeptide antibody (for example, produced as described in Ausubel et al., *supra*, or by any standard technique) may be attached to a column and used to isolate the polypeptide. Lysis and fractionation of SNI1-producing cells prior to affinity

chromatography may be performed by standard methods (see, for example, Ausubel et al., *supra*). Once isolated, the recombinant protein can, if desired, be further purified, for example, by high performance liquid chromatography (see, for example, Fisher, *Laboratory Techniques In Biochemistry And Molecular Biology*, eds., Work and Burdon, Elsevier, 1980).

Polypeptides of the invention, particularly short polypeptide fragments, can also be produced by chemical synthesis (for example, by the methods described in *Solid Phase Peptide Synthesis*, 2nd ed., 1984 The Pierce Chemical Co., Rockford, IL).

Engineering Disease Resistance

As is discussed above, plants having decreased expression of a negative regulator polypeptide (for example, SNI1) are useful, for example, for activating a plant's SAR defense pathways that confer anti-pathogenic properties. Plants having decreased expression of such SAR negative regulator polypeptides are generated according to standard gene silencing methods including, without limitation, co-suppression and antisense methodologies, expression of dominant negative gene products, and creation of plants having mutated genes encoding a negative regulator polypeptide of SAR.

Co-Suppression

One preferred method of silencing gene expression is co-suppression (also referred to as sense suppression). This technique, which involves introduction of a nucleic acid configured in the sense orientation, has been shown to effectively block the transcription of target genes (see for example, Napoli et al., *Plant Cell*, 2:279-289, 1990 and Jorgensen et al., U.S. Patent No. 5,034,323).

Generally, sense suppression involves transcription of the introduced sequence. However, co-suppression may also occur where the introduced sequence contains no coding sequence per se, but only intron or untranslated sequences homologous to sequences present in the primary transcript of the endogenous gene to be repressed. The introduced sequence generally will be substantially identical to the

endogenous gene targeted for repression. Such identity is typically greater than about 50%, but higher identities (for example, 80% or even 95% are preferred because they result in more effective repression. The effect of co-suppression may also be applied to other proteins within a similar family of genes exhibiting homology or substantial homology. Segments from a gene from one plant can be used directly, for example, to inhibit expression of homologous genes in different plant species.

In sense suppression, the introduced sequence, requiring less than absolute identity, need not be full length, relative to either the primary transcription product or to fully processed mRNA. A higher identity in a shorter than full length sequence compensates for a longer, sequence of lesser identity. Furthermore, the introduced sequence need not have the same intron or exon pattern, and identity of non-coding segments may be equally effective. Squences of at least 50 base pairs are preferred, with introduced sequences of greater length being more preferred (see, for example, those methods described by Jorgensen et al., U.S. Patent No. 5,034,323).

In one working example, constitutive sense expression of the *SNI1* gene of Arabidopsis or a *SNI1* homolog may be expressed in Russet Burbank potato to control *Phytophthora infestans* infection. In one particular approach, a plant expression vector is constructed that contains a *SNI1* cDNA sequence expressed under the control of the enhanced CaMV 35S promoter as described by McPherson and Kay (U.S. Patent 5,359,142). This expression vector is then used to transform Russet Burbank according to the methods described in Fischhoff et al. (U.S. Patent 5,500,365). To assess resistance to fungal infection, transformed Russet Burbank and appropriate controls are grown to approximately eight-weeks-old, and leaves (for example, the second or third from the top of the plant) are inoculated with a mycelial suspension of *P. infestans*. Plugs of *P. infestans* mycelia are inoculated on each side of the leaf midvein. Plants are subsequently incubated in a growth chamber at 27°C with constant fluorescent light.

Leaves of transformed Russet Burbank and control plants are then evaluated for resistance to *P. infestans* infection according to conventional experimental methods. For this evaluation, the number of lesions per leaf and percentage of leaf

area infected are recorded every twenty-four hours for seven days after inoculation. From these data, levels of resistance to *P. infestans* are determined. Transformed potato plants that have an increased level of resistance to *P. infestans* relative to control plants are taken as being useful in the invention.

Alternatively, to assess resistance at the whole plant level, transformed and control plants are transplanted to potting soil containing an inoculum of *P. infestans*. Plants are then evaluated for symptoms of fungal infection (for example, wilting or decayed leaves) over a period of time lasting from several days to weeks. Again, transformed potato plants expressing the *SNI1* gene that co-suppress endogenous gene expression having an increased level of resistance to the fungal pathogen, *P. infestans*, relative to control plants are taken as being useful in the invention.

In another working example, expression of the *SNI1* gene of Arabidopsis or a *SNI1* homolog in tomato is used to control bacterial infection, for example, to *Pseudomonas syringae*. Specifically, a plant expression vector is constructed that contains the *SNI1* sequence, in sense orientation, which is expressed under the control of the enhanced CaMV 35S promoter as described by McPherson and Kay, *supra*. This expression vector is then used to transform tomato plants according to the methods described in Fischhoff et al., *supra*. To assess resistance to bacterial infection, transformed tomato plants and appropriate controls are grown, and their leaves are inoculated with a suspension of *P. syringae* according to standard methods, for example, those described herein. Plants are subsequently incubated in a growth chamber, and the inoculated leaves are subsequently analyzed for signs of disease resistance according to standard methods. For example, the number of chlorotic lesions per leaf and percentage of leaf area infected are recorded and evaluated after inoculation. From a statistical analysis of these data, levels of resistance to *P. syringae* are determined. Transformed tomato plants having an increased level of resistance to *P. syringae* relative to control plants are taken as being useful in the invention.

Antisense Suppression

In antisense technology, a nucleic acid segment from the desired plant gene is cloned and operably linked to an expression control region such that the antisense strand of RNA is synthesized. The construct is then transformed into plants and the antisense strand of RNA is produced. In plant cells, it has been shown that antisense RNA inhibits gene expression.

The nucleic acid segment to be introduced in antisense suppression is generally substantially identical to at least a portion of the endogenous gene or genes to be repressed, but need not be identical. The vectors of the present invention therefore can be designed such that the inhibitory effect applies to other proteins within a family of genes exhibiting homology or substantial homology to the target gene. Segments from a gene from one plant can be used, for example, directly to inhibit expression of homologous genes in different plant species.

The introduced sequence also need not be full length relative to either the primary transcription product or to fully processed mRNA. Generally, higher homology can be used to compensate for the use of a shorter sequence. Moreover, the introduced sequence need not have the same intron or exon pattern, and homology of non-coding segments will be equally effective. In general, such an antisense sequence will usually be at least 15 base pairs, preferably about 15-200 base pairs, and more preferably 200-2,000 base pairs in length or greater. The antisense sequence may be complementary to all or a portion of the gene to be suppressed (for example, *SNI1*), and, as appreciated by those skilled in the art, the particular site or sites to which the antisense sequence binds as well as the length of the antisense sequence will vary, depending upon the degree of inhibition desired and the uniqueness of the antisense sequence. A transcriptional construct expressing a plant negative regulator antisense nucleotide sequence includes, in the direction of transcription, a promoter, the sequence coding for the antisense RNA on the sense strand, and a transcriptional termination region. Antisense sequences may be constructed and expressed as described, for example, in van der Krol et al. (*Gene* 72: 45-50, 1988); Rodermel et al. (*Cell* 55: 673-681, 1988); Mol et al. (*FEBS Lett.* 268: 427-430, 1990); Weigel and

Nilsson (*Nature* 377: 495-500, 1995); Cheung et al., (*Cell* 82: 383-393, 1995); and Shewmaker et al. (U.S. Pat. No. 5,107,065).

In one working example, antisense expression of the *SNI1* gene of *Arabidopsis* or a *SNI1* homolog is used to control fungal diseases, for example, the infection of tissue by *Magnaporthe grisea*, the cause of rice blast. In one particular approach, a plant expression vector is constructed that contains the cDNA sequence of *SNI1* homolog in antisense orientation that is constitutively expressed under the control of the rice actin promoter described by Wu et al. (WO 91/09948). This expression vector is then used to transform rice plants according to conventional methods, for example, using the methods described in Hiei et al. (*Plant Journal* 6:271-282, 1994). To assess resistance to fungal infection, transformed rice plants expressing antisense sequences and appropriate controls are grown, and their leaves are inoculated with a mycelial suspension of *M. grisea* according to standard methods. Plants are subsequently incubated in a growth chamber and the inoculated leaves are subsequently analyzed for disease resistance according to standard methods. For example, the number of lesions per leaf and percentage of leaf area infected are recorded and evaluated after inoculation. From a statistical analysis of these data, levels of resistance to *M. grisea* are determined. Transformed rice plants that express antisense *SNI1* RNA having an increased level of resistance to *M. grisea* relative to control plants are taken as being useful in the invention.

Dominant Negatives

Transgenic plants expressing a transgene encoding a dominant negative gene product of a negative regulator polypeptide of SAR are assayed in artificial environments or in the field to demonstrate that the transgene confers disease resistance on the transgenic plant. Dominant negative transgenes are constructed according to methods known in the art. Typically, a dominant negative gene encodes a mutant negative regulator polypeptide of SAR (for example, a mutated SNI1) which, when overexpressed, disrupts the activity of the wild type negative regulator polypeptide. Transgenic plants having an increased ability to grow in the presence of

a plant pathogen, as compared to non-transgenic plants, are useful in the invention.

Mutants

Plants having decreased expression of a negative regulator polypeptide of SAR are also generated using standard mutagenesis methodologies. Such mutagenesis methods include, without limitation, treatment of seeds with ethyl methylsulfate or fast neutron irradiation, as well as T-DNA insertion methodologies. Expression of a negative regulator polypeptide of SAR and disease resistance phenotypes in mutated and non-mutated lines are evaluated according to standard procedures (for example, those described herein). When compared to non-mutated plants, mutated plants having decreased expression of a gene encoding a negative regulator polypeptide of SAR (for example, a *SNI1* homolog) exhibit increased disease resistance relative to their non-mutated counterparts.

Identification and Application of Molecules that Modulate Negative Regulator Polypeptide Expression

Isolation of the *SNI1* cDNA also facilitates the identification of molecules that increase or decrease SNI1 expression. According to one approach, candidate molecules are added at varying concentrations to a culture medium of cells (for example, prokaryotic cells such as *E. coli or* eukaryotic cells such as yeast, mammalian, insect, or plant cells*)* expressing *SNI1* mRNA. *SNI1* expression is then measured, for example, by standard northern blot analysis (Ausubel et al, *supra*) using a *SNI1* cDNA (or cDNA fragment) as a hybridization probe. The level of *SNI1* expression in the presence of the candidate molecule is compared to the level measured for the same cells in the same culture medium but in the absence of the candidate molecule.

If desired, the effect of candidate modulators on expression may, in the alternative, be measured at the level of SNI1 protein production using the same general approach and standard immunological detection techniques, such as western blotting or immunoprecipitation with a *SNI1*-specific antibody.

Candidate modulators may be purified (or substantially purified) molecules or may be one component of a mixture of compounds. In a mixed compound assay, *SNI1* expression is tested against progressively smaller subsets of the candidate compound pool (for example, produced by standard purification techniques, for example, HPLC) until a single compound or minimal compound mixture is demonstrated to modulate *SNI1* gene expression.

A molecule that promotes a decrease in *SNI1* expression is considered particularly useful in the invention; such a molecule may be used, for example, as a chemical regulator to increase a plant's resistance to a pathogen.

Modulators found to be effective at the level of *SNI1* expression or activity may be confirmed as useful *in planta* and, if successful, may be used as anti-pathogen compounds in the field.

For agricultural uses, the molecules, compounds, or agents identified using the methods disclosed herein may be used as chemicals applied as sprays or dusts on the foliage of plants. Typically, such agents are to be administered on the surface of the plant in advance of the pathogen in order to prevent infection. Seeds, bulbs, roots, and tubers are also treated to prevent pathogenic attack after planting by controlling pathogens carried on them or existing in the soil at the planting site. Soil to be planted with vegetables, ornamentals, shrubs, or trees can also be treated with chemical fumigants for control of a variety of microbial pathogens. Treatment is preferably done several days or weeks before planting. The chemicals can be applied by either a mechanized route, for example, a tractor or with hand applications. The molecules, compounds, or agents may also be applied to plants in combination with another molecule which affords some benefit to the plant.

Enhanced Sensitivity to Chemical Regulators of SAR

Because *sni* plants have been found to have enhanced sensitivity to exogenously applied INA as well as enhanced disease resistance relative to wild type and *npr1* plants (Figure 6), the invention also includes methods of increasing the resistance of a plant having decreased expression of a negative regulator polypeptide

of SAR through the exogenous application of chemical regulators of SAR. By "enhanced sensitivity" is meant that the developmental, physiological, or molecular processes that are typically regulated or controlled during SAR exhibit increased or elevated responses to the presence of an exogenously applied chemical regulator relative to those responses in a wild-type plant.

Analysis of a plant's sensitivity to a chemical regulator is accomplished using a wide variety of bioassays. These assays include, but are not limited to, evaluating and monitoring gene expression (for example, of *PR* genes) and resistance to pathogen attack and disease. By comparing phenotypes of wild-type plants and candidate plants (for example, a plant having decreased expression of a negative regulator polypeptide of SAR such as *sni*), one is readily able to determine whether such a candidate plant has an increased sensitivity to a chemical regulator of SAR. Thus, in plants exhibiting an increased sensitivity to an SAR chemical regulator, the aforementioned *PR*-inducible genes have an increased, elevated, or heightened level of chemical-mediated expression. For example, when such chemical regulators are applied to plant tissue, typically to the leaves of whole plants, levels of *PR* mRNA and proteins develop in the plant tissue which are increased relative to wild-type levels. Exemplary chemical regulators useful in the methods of the invention include chemicals known to be inducers of PR proteins in plants, or close derivatives thereof. These include benzoic acid, salicylic acid, BTH, INA, and derivatives and analogs thereof. In addition, plants having enhanced sensitivity to a chemical regulator of SAR require the application of lower concentrations of such regulators for promoting increased levels of disease resistance than control plants.

Interacting Polypeptides

The isolation of sequences encoding a negative regulator polypeptide of SAR also facilitates the identification of polypeptides which interact with other polypeptides involved in SAR. Such polypeptide-encoding sequences are isolated by any standard two hybrid system (see, for example, Fields et al., *Nature* 340:245-246, 1989; Yang et al., *Science* 257:680-682, 1992; Zervos et al., Cell 72:223-232, 1993).

For example, all or a part of the *SNI1* sequence may be fused to a DNA binding domain (such as the GAL4 or LexA DNA binding domain). After establishing that this fusion protein does not itself activate expression of a reporter gene bearing appropriate DNA binding sites (for example, a lacZ or LEU2 reporter gene), this fusion protein is used as an interaction target. Candidate interacting proteins fused to an activation domain (for example, an acidic activation domain) are then co-expressed with the *SNI1* fusion in host cells, and interacting proteins are identified by their ability to contact the *SNI1* sequence and stimulate reporter gene expression. *SNI1*-interacting proteins identified using this screening method provide good candidates for proteins that are involved in the systemic acquired resistance signal transduction pathway.

Antibodies

The polypeptides described herein (or imunogenic fragments or analogs) may be used to raise antibodies useful in the invention; such polypeptides may be produced by recombinant or peptide synthetic techniques (see, for example, *Solid Phase Peptide Synthesis*, 2nd ed., 1984, Pierce Chemical Co., Rockford, IL; Ausubel et al., *supra*). The peptides may be coupled to a carrier protein, such as KLH as described in Ausubel et al, *supra*. The KLH-peptide is mixed with Freund's adjuvant and injected into guinea pigs, rats, or preferably rabbits. Antibodies may be purified by peptide antigen affinity chromatography.

Monoclonal antibodies may be prepared using any of the polypeptides described above and standard hybridoma technology (see, for example, Hammerling et al., *In Monoclonal Antibodies and T Cell Hybridomas*, Elsevier, NY, 1981; Ausubel et al., *supra*; Harlow and Lane, *Antibodies- A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y. 1988).

Once produced, polyclonal or monoclonal antibodies are tested for specific recognition of an antigen by western blot or immunoprecipitation analysis (by the methods described in Ausubel et al., *supra*). Antibodies which specifically recognize SAR negative regulator polypeptides (for example, SNI1) are considered to be useful

in the invention; such antibodies may be used, for example, in an immunoassay to monitor the level of such a polypeptide produced by a plant.

Use

The invention described herein is useful for a variety of agricultural and commercial purposes including, but not limited to, improving systemic acquired resistance against plant pathogens, increasing crop yields, improving crop and ornamental quality, and reducing agricultural production costs. In particular, silencing or decreasing the expression of a negative regulator polypeptide of SAR (for example, *SNI1*) in a plant cell provides a method for activating systemic acquired resistance to plant pathogens and can be used to protect plants from pathogen infestation that reduces plant productivity and viability.

The invention also provides for broad-spectrum pathogen resistance by facilitating the natural mechanism of host resistance. For example, sense and antisense transgenes or both can be expressed in plant cells at sufficiently high levels to silence the expression of a negative regulator polypeptide (for example, SNI1) thereby turning on a systemic resistance plant defense response constitutively in the absence of signals from the acquired pathogen. The level of expression associated with such a plant defense response may be determined by measuring the levels of defense response gene expression as described herein or according to any conventional method. If desired, the transgenes are expressed by a controllable promoter such as a tissue-specific promoter, cell-type specific promoter, or by a promoter that is induced by an external signal or agent such as a pathogen- or wound-inducible control element, thus limiting the temporal or tissue expression or both of a systemic acquired resistance defense response. If desired, the genes designed to silence the expression of a negative regulator polypeptide of SAR may also be expressed in roots, leaves, or fruits, or at a site of a plant that is susceptible to pathogen penetration and infection.

The invention is also useful for controlling plant disease by enhancing a plant's SAR defense mechanisms. In particular, the invention is useful for combating diseases known to be inhibited by plant SAR defense mechanisms. These include, without limitation, viral diseases caused by TMV and TNV, bacterial diseases caused by *Pseudomonas* and *Xanthomonas*, and fungal diseases caused by *Erysiphe*, *Peronospora*, *Phytophthora*, *Colletotrichum*, and *Magnaporthe grisea*. In particular exemplary approaches, constitutive or inducible expression of a gene encoding a negative regulator polypeptide of SAR (for example, in sense or antisense orientation) in a transgenic plant is useful for controlling powdery mildew of wheat caused by *Erysiphe*, bacterial leaf spot of pepper caused by *Xanthomonas campestris*, bacterial wilt and bacterial spot of tomato caused by *Pseudomonas syringae* and *Xanthomonas campestris*, and bacterial blights of citrus and walnut caused by *Xanthomonas campestris*.

Other Embodiments

The invention further includes analogs of any naturally-occurring plant negative regulator polypeptide of SAR (for example, SNI1). Analogs can differ from the naturally-occurring polypeptide by amino acid sequence differences, by post-translational modifications, or by both. Analogs of the invention will generally exhibit at least 40%, more preferably 50%, and most preferably 60% or even having 70%, 80%, or 90% identity with all or part of a naturally-occurring plant negative regulator polypeptide amino acid sequence. The length of sequence comparison is at least 15 amino acid residues, preferably at least 25 amino acid residues, and more preferably more than 35 amino acid residues. Modifications include *in vivo* and *in vitro* chemical derivatization of polypeptides, for example, acetylation, carboxylation, phosphorylation, or glycosylation; such modifications may occur during polypeptide synthesis or processing or following treatment with isolated modifying enzymes. Analogs can also differ from the naturally-occurring negative regulator polypeptide (for example, SNI1) by alterations in primary sequence. These include genetic variants, both natural and induced (for example, resulting from random mutagenesis

by irradiation or exposure to ethyl methylsulfate or by site-specific mutagenesis as described in Sambrook et al., *supra*, or Ausubel et al., *supra*). Also included are cyclized peptides, molecules, and analogs which contain residues other than L-amino acids, for example, D-amino acids or non-naturally occurring or synthetic amino acids, for example, β or γ amino acids.

In addition to full-length polypeptides, the invention also includes negative regulator polypeptide fragments. As used herein, the term "fragment," means at least 20 contiguous amino acids, preferably at least 30 contiguous amino acids, more preferably at least 50 contiguous amino acids, and most preferably at least 60 to 80 or more contiguous amino acids. Fragments of negative regulator polypeptides (for example, SNI1) can be generated by methods known to those skilled in the art or may result from normal protein processing (for example, removal of amino acids from the nascent polypeptide that are not required for biological activity or removal of amino acids by alternative mRNA splicing or alternative protein processing events). In preferred embodiments, a negative regulator polypeptide fragment includes an N domain motif (for example, such as a domain that is substantially identical to the N domain of the retinoblastoma protein (SEQ ID NO:5) shown in Fig. 3C) as described herein.

Furthermore, the invention includes nucleotide sequences that facilitate specific detection of a gene encoding a negative regulator polypeptide of SAR. Thus, the nucleic acid sequences described herein or portions thereof may be used as probes to hybridize to nucleotide sequences from other plants (for example, dicots, monocots, gymnosperms, and algae) by standard hybridization techniques under conventional conditions. Sequences that hybridize to a negative regulator polypeptide coding sequence or its complement and that encode a negative regulator polypeptide are considered useful in the invention. As used herein, the term "fragment," as applied to nucleic acid sequences, means at least 5 contiguous nucleotides, preferably at least 10 contiguous nucleotides, more preferably at least 20 to 30 contiguous nucleotides, and most preferably at least 40 to 80 or more contiguous nucleotides. Fragments of negative regulator polypeptide nucleic acid sequences (for example, *SNI1*) can be

generated by methods known to those skilled in the art.

All publications and patent applications mentioned in this specification are herein incorporated by reference.

Other embodiments are with the claims.

What is claimed:

Claims

1. A non-naturally occurring plant having decreased expression of an endogenous negative regulator polypeptide of systemic acquired resistance (SAR), wherein said decreased expression renders said non-naturally occurring plant resistant to a plant pathogen.

2. The non-naturally occurring plant of claim 1, wherein said plant is a transgenic plant.

3. The non-naturally occurring plant of claim 2, wherein said transgenic plant comprises a transgene that, when expressed in said transgenic plant, silences gene expression of said endogenous negative regulator polypeptide of SAR; a transgene that expresses an antisense molecule of said negative regulator polypeptide of SAR; or a transgene that, when expressed in said transgenic plant, co-suppresses expression of said negative regulator polypeptide of SAR or both.

4. The non-naturally occurring plant of claim 2, wherein said transgenic plant comprises a transgene that encodes a dominant negative gene product.

5. The non-naturally occurring plant of claim 4, wherein said dominant negative gene product comprises a gene that encodes a mutated form of said negative regulator polypeptide of SAR.

6. The non-naturally occurring plant of claim 1, wherein said plant comprises a point mutation, a deletion, or an insertion in a gene that encodes said negative regulator polypeptide of SAR.

7. The non-naturally occurring plant of claim 1, wherein said decreased expression of said negative regulator polypeptide of SAR occurs at the transcriptional level, the translational level, or post-translational level.

8. The non-naturally occurring plant of claim 1, wherein said negative regulator polypeptide of SAR has at least 30% identity to the amino acid sequence shown in Fig. 3A.

9. An isolated nucleic acid molecule comprising a sequence encoding a negative regulator polypeptide of SAR.

10. The isolated nucleic acid molecule of claim 9, wherein said sequence encodes a negative regulator polypeptide of SAR having at least 30% identity to the amino acid sequence shown in Fig. 3A.

11. The isolated nucleic acid molecule of claim 9, wherein said sequence encodes a negative regulator polypeptide of SAR that is leucine-rich; has at least 20% sequence identity to the N domain of a retinoblastoma protein; that represses transcription, or that is substantially localized to the nucleus.

12. The nucleic acid molecule of claim 9, wherein said nucleic acid molecule is cDNA.

13. The isolated nucleic acid molecule of claim 9, wherein said isolated nucleic acid molecule hybridizes specifically to a nucleic acid molecule comprising the sequence of Fig. 3A or 3B.

14. The isolated nucleic acid molecule of claim 9, wherein said isolated nucleic acid molecule is operably linked to a promoter functional in a plant cell.

15. An expression vector comprising the isolated nucleic acid molecule of claim 9, said vector being capable of directing expression of the polypeptide encoded by said isolated nucleic acid molecule.

16. A cell comprising the isolated nucleic acid molecule of claim 9.

17. The cell of claim 16, wherein said cell is a plant cell.

18. The cell of claim 16, wherein said cell is a bacterial cell.

19. The cell of claim 16, wherein said bacterial cell is *Agrobacterium*.

20. A transgenic plant or transgenic plant component comprising the nucleic acid molecule of claim 9, wherein said nucleic acid molecule is expressed in said transgenic plant or said transgenic plant component.

21. The transgenic plant or transgenic plant component of claim 20, wherein said transgenic plant or transgenic plant component is from an angiosperm, a dicot, a monocot, or a crucifer.

22. A seed from the transgenic plant or transgenic plant component of claim 20.

23. An isolated DNA molecule encoding an antisense RNA of a negative regulator polypeptide of SAR.

24. An expression vector comprising the DNA molecule of claim 23.

25. A transgenic plant or transgenic plant component comprising the vector of claim 24.

26. A seed from the transgenic plant or transgenic plant component of claim 25.

27. A cell from the transgenic plant or transgenic plant component of claim 25.

28. A substantially pure negative regulator polypeptide of SAR.

29. The substantially pure polypeptide of claim 28, said polypeptide comprising an amino acid sequence having at least 30% identity to the amino acid sequence of Fig. 3A or having at least 20% identity to the N domain of a retinoblastoma protein.

30. The substantially pure polypeptide of claim 28, wherein said polypeptide comprises an amino acid sequence that is leucine rich, represses transcription, or is predominately localized to the nucleus.

31. A method of producing a negative regulator polypeptide of SAR, said method comprising the steps of:

(a) providing a cell transformed with the isolated nucleic acid molecule of claim 9;

(b) culturing said transformed cell under conditions for expressing said isolated nucleic acid molecule; and

(c) recovering said negative regulator polypeptide of SAR.

32. A recombinant negative regulator polypeptide of SAR produced according to the method of claim 31.

33. A substantially pure antibody that specifically recognizes and binds to a negative regulator polypeptide of SAR.

34. The antibody of claim 33, wherein said antibody recognizes and binds to a recombinant negative regulator polypeptide of SAR.

35. A method of isolating a negative regulator gene of SAR or fragment thereof, said method comprising the steps of:

(a) contacting the nucleic acid molecule of Fig. 3A or a portion thereof with a nucleic acid preparation from a plant cell under hybridization conditions providing detection of nucleic acid sequences having at least 30% or greater sequence identity to the nucleic acid sequence of Fig. 3A; and

(b) isolating said hybridizing nucleic acid sequences.

36. A method of isolating a negative regulator gene of SAR or fragment thereof, said method comprising the steps of:

(a) providing a sample of plant cell DNA;

(b) providing a pair of oligonucleotides having sequence identity to a region of the nucleic acid molecule of Fig. 3A;

(c) contacting the pair of oligonucleotides with said plant cell DNA under conditions suitable for polymerase chain reaction-mediated DNA amplification; and

(d) isolating the amplified negative regulator gene of SAR or fragment thereof.

37. The method of claim 36, wherein said amplification step is carried out using a sample of cDNA prepared from a plant cell.

38. The method of claim 36, wherein said negative regulator gene of SAR encodes a polypeptide which is at least 30% identical to the amino acid sequence of Fig. 3A.

39. A process for conferring disease resistance on a transgenic plant or transgenic plant component, said method comprising the steps of:

(a) introducing into plant cells a transgene encoding a negative regulator polypeptide of SAR operably linked to a promoter functional in said plant cells to yield transformed plant cells; and

(b) regenerating a transgenic plant or transgenic plant component from said transformed plant cells, wherein the negative regulator polypeptide of SAR is expressed in the cells of said transgenic plant or transgenic plant component, thereby conferring disease resistance on said transgenic plant or transgenic plant component.

40. The method of claim 39, wherein expression of said transgene cosuppresses the expression of an endogenous negative regulator of SAR.

41. The method of claim 39, wherein said transgene encoding said negative regulator polypeptide of SAR is constitutively or inducibly expressed.

42. The method of claim 39, wherein said transgene encoding said negative regulator polypeptide of SAR is expressed in a tissue-specific, cell-specific, or organ-specific manner.

43. A process for conferring disease resistance on a transgenic plant or transgenic plant component, said method comprising the steps of:

(a) introducing into plant cells a transgene encoding an antisense coding sequence of a negative regulator polypeptide of SAR operably linked to a promoter functional in said plant cells to yield transformed plant cells; and

(b) regenerating a transgenic plant or transgenic plant component from said transformed plant cells, wherein the antisense coding sequence of the negative regulator polypeptide of SAR is expressed in the cells of said transgenic plant or transgenic plant component, thereby conferring disease resistance on said transgenic plant or transgenic plant component.

44. The method of claim 43, wherein said transgene encoding an antisense coding sequence of a negative regulator polypeptide of SAR is constitutively or is inducibly expressed.

45. The method of claim 43, wherein said transgene encoding an antisense coding sequence of a negative regulator polypeptide of SAR is expressed in a tissue-specific, cell-specific, or organ-specific manner.

46. A process for conferring disease resistance on a transgenic plant or transgenic plant component, said method comprising the steps of:

(a) introducing into plant cells a transgene encoding a dominant negative gene product of a negative regulator polypeptide of SAR operably linked to a promoter functional in said plant cells to yield transformed plant cells; and

(b) regenerating a transgenic plant or transgenic plant component from said transformed plant cells, wherein said dominant negative gene product of said negative regulator of SAR is expressed in the cells of said transgenic plant or transgenic plant component, thereby conferring disease resistance on said transgenic plant or transgenic plant component.

47. The method of claim 46, wherein said transgene encoding said dominant negative gene product is constitutively or is inducibly expressed.

48. The method of claim 46, wherein said transgene encoding said dominant negative gene product of a negative regulator polypeptide of SAR is expressed in a tissue-specific, cell-specific, or organ-specific manner.

49. A method for conferring disease resistance on a transgenic plant cell, said method comprising reducing the level of an endogenous negative regulator polypeptide of SAR in a transgenic plant cell.

50. The method of claim 49, wherein said transgenic plant cell is from a monocot, a dicot, or a gymnosperm.

51. The method of claim 49, wherein reducing the level of said endogenous negative regulator polypeptide of SAR comprises expressing a transgene encoding an antisense nucleic acid molecule of a negative regulator gene of SAR in said transgenic plant cell.

52. The method of claim 49, wherein reducing the level of said endogenous negative regulator polypeptide of SAR comprises co-suppression of said endogenous negative regulator gene of SAR in said transgenic plant cell.

53. The method of claim 49, wherein reducing the level of said endogenous negative regulator polypeptide of SAR comprises expressing a dominant negative gene product in said transgenic plant cell.

54. The method of claim 53, wherein said dominant negative gene product is a mutated form of said endogenous negative regulator polypeptide of SAR.

55. A method for identifying a compound which modulates the expression of a gene encoding a negative regulator polypeptide of SAR in a cell, said method comprising the steps of:

(a) providing a cell comprising a gene encoding a negative regulator polypeptide of SAR;

(b) applying to said cell a candidate compound; and

(c) measuring expression of said gene encoding said negative regulator polypeptide of SAR, an increase or decrease in expression relative to an untreated control sample being an indication that said compound modulates expression of said negative regulator polypeptide of SAR.

56. The method according to claim 55, wherein said gene encodes a negative regulator polypeptide of SAR having at least 30% identity to the amino acid sequence shown in Fig. 3A.

57. The method of claim 55, wherein said compound decreases the expression of said gene that encodes said negative regulator polypeptide of SAR.

58. The method of claim 55, wherein said compound increases the expression of said gene that encode said negative regulator polypeptide of SAR.

59. A method for identifying a compound which modulates the activity of a negative regulator polypeptide of SAR in a cell, said method comprising the steps of:

(a) providing a cell expressing a gene encoding a negative regulator polypeptide of SAR;

(b) applying to said cell a candidate compound; and

(c) measuring the activity of said negative regulator polypeptide of SAR, an increase or decrease in activity relative to an untreated control sample being an indication that said compound modulates activity of said negative regulator polypeptide of SAR.

60. The method according to claim 59, wherein said gene encodes a negative regulator polypeptide of SAR having at least 30% identity to the amino acid sequence shown in Fig. 3A.

61. The method of claim 59, wherein said compound decreases said activity of said negative regulator polypeptide of SAR.

62. The method of claim 59, wherein said compound increases said activity of said negative regulator polypeptide of SAR.

63. A method for increasing the resistance of a plant or plant component to a pathogen, said method comprising applying a compound to said plant or plant component that decreases the expression of a gene encoding a negative regulator polypeptide of SAR.

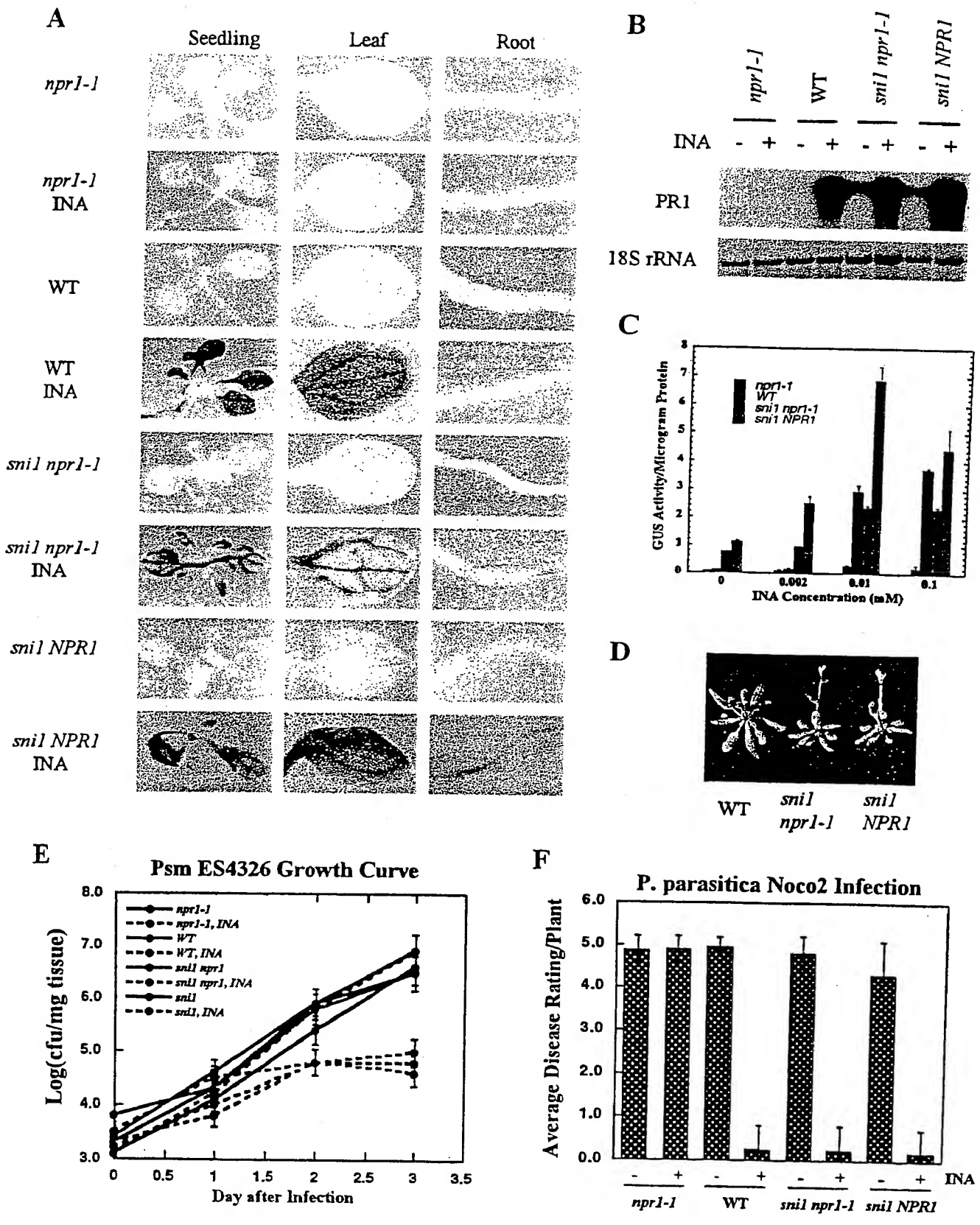
64. A method for increasing the resistance of a plant or plant component to a pathogen, said method comprising applying a compound to said plant or plant component that decreases activity of a negative regulator polypeptide of SAR.

65. A method for increasing the resistance of a plant or plant component to a pathogen, said method comprising applying a chemical regulator of SAR to a plant or plant component that has decreased expression of a negative regulator polypeptide of SAR.

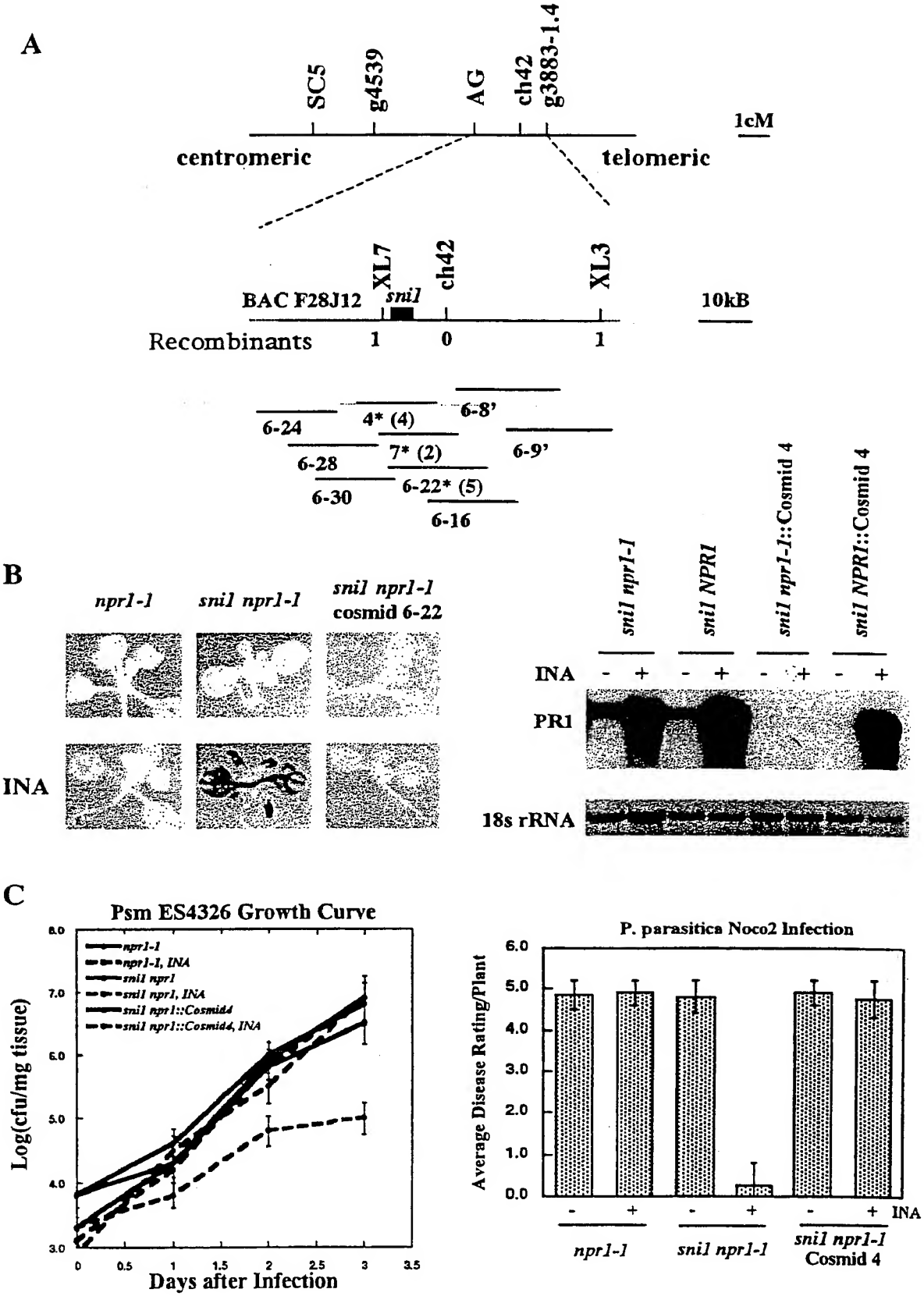
66. The method of claim 65, wherein said chemical inducer of SAR is SA.

67. The method of claim 65, wherein said chemical inducer of SAR is INA.

68. The method of claim 65, wherein said chemical inducer of SAR is BTH.



FIGURES 1A-1F



FIGURES 2A-2C

A

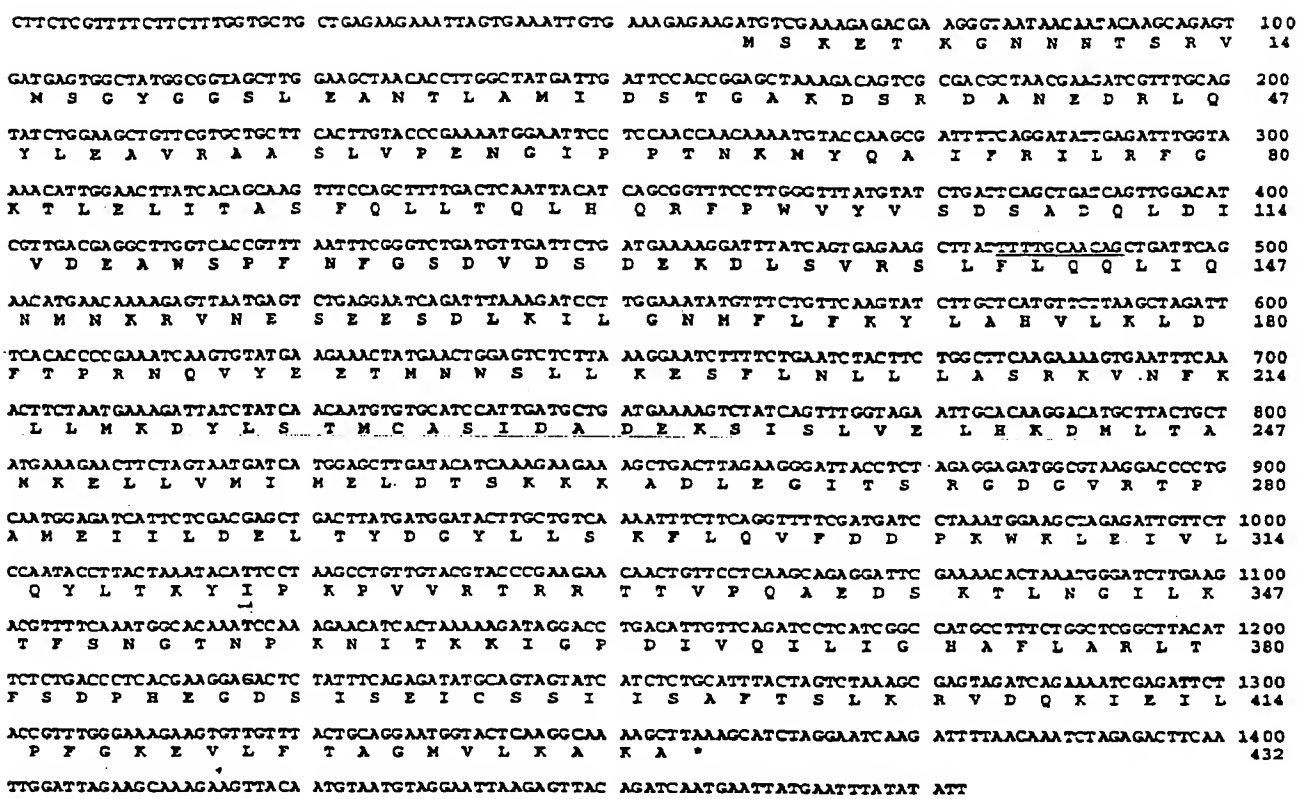


FIGURE 3A

SNI1 genomic sequence
The exons are highlighted.
The start and stop codons are underlined.

Sequence Range: 1 to 5040

```
                                                50                                                  100
        *         *         *         *         *         *         *         *         *         *
GCTTCCTCTGCAGTTTGGAACGTTC CGAGCCAAACCCTAACACCGTTTCT CGTCGAATCCCTGATTTCGGCTGCA AATTTCCCCCACGGACGCTTCCGTA
CGAAGGAGACGTCAAACCTTGCAAG GCTCGGTTTGGGATTGTGGCAAAGA GCAGCTTAGGGACTAAAGCCGACGT TTAAAGGGGGTGCCTGCGAAGGCAT

                                               150                                                  200
        *         *         *         *         *         *         *         *         *         *
CTCCCCTGTATTTTCTTGTTTTGCA AGAAACCCGAGAAGACAAAGAACAT GAATCAATGGTAGGGTTGGTTAATG GATCATGATTGGTTTTGTTTGGGAA
GAGGGGACATAAAAGAACAAAACGT TCTTTGGGCTCTTCTGTTTCTTGTA CTTAGTTACCATCCCAACCAATTAC CTAGTACTAACCAAAACAAACCCTT

                                               250                                                  300
        *         *         *         *         *         *         *         *         *         *
AGAAGCAATAGCATCGAGAATCTCT GTCCATTCCTGGTCCTCCGTCGACA TATCTAGGCTTTGAAGCATTTCTCT AGAACCTTCTGGTATGTCATCAAGG
TCTTCGTTATCGTAGCTCTTAGAGA CAGGTAAGGACCAGGAGGCAGCTGT ATAGATCCGAAACTTCGTAAAGAGA TCTTGGAAGACCATACAGTAGTTCC

                                               350                                                  400
        *         *         *         *         *         *         *         *         *         *
GTAGGCAACGCTTCCCAAGTTCTAG AAACTTCTGGAACTTGAATTGATCT TGTATCGGTTTGTGGTGTTCCGATG TAATTCGCCCAAACGTTCTTTAGGA
CATCCGTTGCGAAGGGTTCAAGATC TTTGAAGACCTTGAACTTAACTAGA ACATAGCCAAACACCACAAGGCTAC ATTAAGCGGGTTTGCAAGAAATCCT

                                               450                                                  500
        *         *         *         *         *         *         *         *         *         *
TCTCGCCGTCTAGTTCTTGGATATT GCCAAAAGCCATTGTTGAATCAGAG AGGAGTATGTGTTGGTGAGATTTAG GGCATTTTAAAGAGATTGTTGCTTT
AGAGCGGCAGATCAAGAACCTATAA CGGTTTTCGGTAACAACTTAGTCTC TCCTCATACACAACCACTCTAAATC CCGTAAAATTTCTCTAACAACGAAA

                                               550                                                  600
        *         *         *         *         *         *         *         *         *         *
GGGGGTTTTGTGTGTACGTTGAATG GGAAATCTGCGGCAGGATAATTGGG AAGTACAGTTGGTGAAACTTGTGTT CTAAGGTACTGACCGCATTTCATGG
CCCCCAAAACACACATGCAACTTAC CCTTTAGACGCCGTCCTATTAACCC TTCATGTCAACCACTTTGAACACAA GATTCCATGACTGGCGTAAAGTACC

                                               650                                                  700
        *         *         *         *         *         *         *         *         *         *
AGGGTTTTAGGTTCCACATAAAATC AGGGGTTTTCTGTGTATACACACAC ACACGTACCATTATGTGAATAGATC ATCTTAAGTCAACTTCAATAAATTA
TCCCAAAATCCAAGGTGTATTTTAG TCCCCAAAAGACACATATGTGTGTG TGTGCATGGTAATACACTTATCTAG TAGAATTCAGTTGAAGTTATTTAAT

                                               750                                                  800
        *         *         *         *         *         *         *         *         *         *
ATTTAATAATGTTACCTCAGTTTGA AGTATAAAGATTGTGTACTATTTTC ATTAAAAAAGTGCAGATTGTGTACT CTTGAAAAGGACTAGATGCAGAGTA
TAAATTATTACAATGGAGTCAAACT TCATATTTCTAACACATGATAAAAG TAATTTTTTCACGTCTAACACATGA GAACTTTTCCTGATCTACGTCTCAT

                                               850                                                  900
        *         *         *         *         *         *         *         *         *         *
TTAATTCACTTTTGTATAATAAACA TATTAATCTTCAGTTTAACGCTTCA TGAGAATCTATGACGTGTTCCCACC AGAGGCACAAATCCGTTTTGGAGAA
AATTAAGTGAAAACATATTATTTGT ATAATTAGAAGTCAAATTGCGAAGT ACTCTTAGATACTGCACAAGGGTGG TCTCCGTGTTTAGGCAAAACCTCTT

                                               950                                                 1000
        *         *         *         *         *         *         *         *         *         *
TTGTGATTATTAAATCAAACATAAG TTTATTGTTTTAATGTTTCGTTCCA CGCTCGTCGCATTTTAAAAAACTTT TTAACTATTTTTTTGGTTATAAAAA
AACACTAATAATTTAGTTTGTATTC AAATAACAAAATTACAAAGCAAGGT GCGAGCAGCGTAAAATTTTTTGAAA AATTGATAAAAAAACCAATATTTTT

                                              1050                                                 1100
        *         *         *         *         *         *         *         *         *         *
GAAATATAGCTTCTAAATGACTTGT TTCTACTGTTAGATTGATGAACCTT AATACTGATTTAGCTTTTCATACAT CGATAAAGAATTCTTCGGTGTTTAG
CTTTATATCGAAGATTTACTGAACA AAGATGACAATCTAACTACTTGGAA TTATGACTAAATCGAAAAGTATGTA GCTATTTCTTAAGAAGCCACAAATC
```

FIGURE 3B

```
                                    1150                                                1200
          *         *         *         *         *         *         *         *         *         *
TTAAGTTTGTTAGAAGTTATTATTT TACCATACGATCACAGTTTAACGCA TATTGCCTAGAGAATATAATATAAT GATAACTAAACGCTATCAAAATGTG
AATTCAAACAATCTTCAATAATAAA ATGGTATGCTAGTGTCAAATTGCGT ATAACGGATCTCTTATATTATATTA CTATTGATTTGCGATAGTTTTACAC

                                    1250                                                1300
          *         *         *         *         *         *         *         *         *         *
AAACGAAATAAGAAAAAACATGTAA GGGCTAAGTCATCACTAACTTTTTA TCCAAGTATTATTTGTATGAGATCA TTTTCCGTAAGTCCAAAGATTTGAA
TTTGCTTTATTCTTTTTTGTACATT CCCGATTCAGTAGTGATTGAAAAAT AGGTTCATAATAAACATACTCTAGT AAAAGGCATTCAGGTTTCTAAACTT

                                    1350                                                1400
          *         *         *         *         *         *         *         *         *         *
ACAATGATGAGAGGTGAATAGAATA TATAAATTCCACACGCTTTGAAACA TAGCTTACAATTGAAAAAAAAATGAA AAAGAGATTAATATCTCTATTCAAT
TGTTACTACTCTCCACTTATCTTAT ATATTTAAGGTGTGCGAAACTTTGT ATCGAATGTTAACTTTTTTTTACTT TTTCTCTAATTATAGAGATAAGTTA

                                    1450                                                1500
          *         *         *         *         *         *         *         *         *         *
GTATTATAGAGAATGAGCTAGAGAT ACTTTATATGGTAGGTTCAAGTCAC CTCAAGTACGTATCAGCTAATATTA AAAAACTATCTGCTAATTTTTATAT
CATAATATCTCTTACTCGATCTCTA TGAAATATACCATCCAAGTTCAGTG GAGTTCATGCATAGTCGATTATAAT TTTTTGATAGACGATTAAAAATATA

                                    1550                                                1600
          *         *         *         *         *         *         *         *         *         *
CAATCGTTTAAGATTCATTCATTCG TTCTCCACTACGAGAGTTTCTTGCT TTGGCACATATATAGTTCAAGAAAG TCTCGTGTTGGAGAGACTCCAAGCG
GTTAGCAAATTCTAAGTAAGTAAGC AAGAGGTGATGCTCTCAAAGAACGA AACCGTGTATATATCAAGTTCTTTC AGAGCACAACCTCTCTGAGGTTCGC

                                    1650                                                1700
          *         *         *         *         *         *         *         *         *         *
TGCTAGAGCGTTTCGATTAGAAAGG TGTTTTGTTCGAGCATTAAAGATTG TTGCAATGGTTCTATCTTAAGGTAG TCATTTGTTAAATTTTGGTATTCAA
ACGATCTCGCAAAGCTAATCTTTCC ACAAAACAAGCTCGTAATTTCTAAC AACGTTACCAAGATAGAATTCCATC AGTAAACAATTTAAAACCATAAGTT

                                    1750                                                1800
          *         *         *         *         *         *         *         *         *         *
TCTCGAGAGCTTCATAAGTCTAGCG AGCTTCATAAACACACTACAAGTCA TGTGTATAAAATTTCCAATTCTGTA ATCACAAGTCTAAACTTAATCTGAA
AGAGCTCTCGAAGTATTCAGATCGC TCGAAGTATTTGTGTGATGTTCAGT ACACATATTTTAAAGGTTAAGACAT TAGTGTTCAGATTTGAATTAGACTT

                                    1850                                                1900
          *         *         *         *         *         *         *         *         *         *
TCCAAATCACAAAAAGCATTTTCAA AATTGATTAAAAAAAAAACTACAAT ATAGAAAAGATTTTAATTGATGCTT GGAACGGAACTAACATTACCTGTTA
AGGTTTAGTGTTTTTCGTAAAAGTT TTAACTAATTTTTTTTTTTGATGTTA TATCTTTTCTAAAATTAACTACGAA CCTTGCCTTGATTGTAATGGACAAT

                                    1950                                                2000
          *         *         *         *         *         *         *         *         *         *
TGATTTCGGGCCTGAAAGTATTCTG AGTTTTGATTCAGAACCACTCGGTA TTAAAGAAAAAGAAGACTTACTACA AAAAAAAAATTATATATATTTTTAT
ACTAAAGCCCGGACTTTCATAAGAC TCAAAACTAAGTCTTGGTGAGCCAT AATTTCTTTTTCTTCTGAATGATGT TTTTTTTTTAATATATATAAAAATA

                                    2050                                                2100
          *         *         *         *         *         *         *         *         *         *
GAATTATTATAACCAACTAATATTG TCTCTATTGGTTCTAAGTTCAAACT TCTAACCTTCGAGGGCCGGGTTCAA ATTCCAGTAAGTGATTTATTTTTTG
CTTAATAATATTGGTTGATTATAAC AGAGATAACCAAGATTCAAGTTTGA AGATTGGAAGCTCCCGGCCCAAGTT TAAGGTCATTCACTAAATAAAAAAC

                                    2150                                                2200
          *         *         *         *         *         *         *         *         *         *
CTAACAATTCTCTTATGGCGGGAAA ATTGACTTCTGTCGAGAATTAGGGT TTCTACAAATCTTTCTCCGTTCTAA TTTCGGATTAAGATTTCAATCTCTC
GATTGTTAAGAGAATACCGCCCTTT TAACTGAAGACAGCTCTTAATCCCA AAGATGTTTAGAAAGAGGCAAGATT AAAGCCTAATTCTAAAGTTAGAGAG

                                    2250                                                2300
          *         *         *         *         *         *         *         *         *         *
CTTCTCGTTTTCTTCTTTGGTGCTG CTGAGAAGAAATTAGTGAAATTGTG AAAGAGAAGATGTCGAAAGAGACGA AGGGTAATAACAATACAAGCAGAGT
GAAGAGCAAAAGAAGAAACCACGAC GACTCTTCTTTAATCACTTTAACAC TTTCTCTTCTACAGCTTTCTCTGCT TCCCATTATTGTTATGTTCGTCTCA
```

FIGURE 3B

```
                                        2350                                                2400
              *         *         *         *         *         *         *         *         *         *
GATGAGTGGCTATGGCGGTAGCTTG GAAGCTAACACCTTGGCTATGATTG ATTCCACCGGAGCTAAAGACAGTCG CGACGCTAACGAAGATCGTACGTTT
CTACTCACCGATACCGCCATCGAAC CTTCGATTGTGGAACCGATACTAAC TAAGGTGGCCTCGATTTCTGTCAGC GCTGCGATTGCTTCTAGCATGCAAA

                                        2450                                                2500
              *         *         *         *         *         *         *         *         *         *
CCTCAATTTTCAATCTTCGTCTTTG TTTTTGTTAATTGTATTGATCCTTT GAATGAAAAACCTTAGGTTTGCAGT ATCTGGAAGCTGTTCGTGCTGCTTC
GGAGTTAAAAGTTAGAAGCAGAAAC AAAAACAATTAACATAACTAGGAAA CTTACTTTTTGGAATCCAAACGTCA TAGACCTTCGACAAGCACGACGAAG

                                        2550                                                2600
              *         *         *         *         *         *         *         *         *         *
ACTTGTACCCGAAAATGGAATTCCT CCAACCAAGTAAAAAATAAAAAACT CTGTTTTTCTAAGAAATTTGAAGTT TTTACCTCTAAAAGAGTACTGATTT
TGAACATGGGCTTTTACCTTAAGGA GGTTGGTTCATTTTTTATTTTTTGA GACAAAAAGATTCTTTAAACTTCAA AAATGGAGATTTTCTCATGACTAAA

                                        2650                                                2700
              *         *         *         *         *         *         *         *         *         *
TGATTTGGGTTTTGTTTAATTTTGA AGCAAAATGTACCAAGCGATTTTCA GGATATTGAGATTTGGTAAAACATT GGAACTTATCACAGCAAGTTTCCAG
ACTAAACCCAAAACAAATTAAAACT TCGTTTTACATGGTTCGCTAAAAGT CCTATAACTCTAAACCATTTTGTAA CCTTGAATAGTGTCGTTCAAAGGTC

                                        2750                                                2800
              *         *         *         *         *         *         *         *         *         *
CTTTTGACTCAATTACATCAGGTAC TTGTTGCAAGATTTTGGTAGTGTAA TTTGTCCAATAACTTTTATGAGGTT TCAGTGTTTTGCTTATTATGATTAT
GAAAACTGAGTTAATGTAGTCCATG AACAACGTTCTAAAACCATCACATT AAACAGGTTATTGAAAATACTCCAA AGTCACAAAACGAATAATACTAATA

                                        2850                                                2900
              *         *         *         *         *         *         *         *         *         *
GTTTTGTTTCAGCGGTTTCCTTGGG TTTATGTATCTGATTCAGCTGATCA GTTGGACATCGTTGACGAGGTACTT GTTACAAAAGTTTTGATGTGTTTTT
CAAAACAAAGTCGCCAAAGGAACCC AAATACATAGACTAAGTCGACTAGT CAACCTGTAGCAACTGCTCCATGAA CAATGTTTTCAAAACTACACAAAAA

                                        2950                                                3000
              *         *         *         *         *         *         *         *         *         *
GTTCATTCTTTGTTATGGTGGGGAT GGTTGATTTCATGTGTTTGTTTATG GTTTTGTTTTGCAGGCTTGGTCACG GTTTAATTTCGGGTCTGATGTTGAT
CAAGTAAGAAACAATACCACCCCTA CCAACTAAAGTACACAAACAAATAC CAAAACAAAACGTCCGAACCAGTGC CAAATTAAAGCCCAGACTACAACTA

                                        3050                                                3100
              *         *         *         *         *         *         *         *         *         *
TCTGATGAAAAGGATTTATCAGTGA GAAGCTTATGTAAGGACGTTTGTCT TCTCTTTTTGAGTTAGCTTTTTGTT GGTTCTTGCTAAATATGATTTTTTG
AGACTACTTTTCCTAAATAGTCACT CTTCGAATACATTCCTGCAAACAGA AGAGAAAAACTCAATCGAAAAACAA CCAAGAACGATTTATACTAAAAAAC

                                        3150                                                3200
              *         *         *         *         *         *         *         *         *         *
TTTGTTTTATGATTTCAGTTTTGCA ACAGCTGATTCAGAACATGAACAAA AGAGTTAATGAGTCTGAGGAATCAG ATTTAAAGGTTGATTTATTTGTTAT
AAACAAAATACTAAAGTCAAAACGT TGTCGACTAAGTCTTGTACTTGTTT TCTCAATTACTCAGACTCCTTAGTC TAAATTTCCAACTAAATAAACAATA

                                        3250                                                3300
              *         *         *         *         *         *         *         *         *         *
GTTGCAGATAAGCTTTAAAAATTGG TAGACTTTCACACAACTTGTTGTTA TTTGATTCTTTTGTTTGGATTTCTG CAGATCCTTGGAAATATGTTTCTGT
CAACGTCTATTCGAAATTTTTAACC ATCTGAAAGTGTGTTGAACAACAAT AAACTAAGAAAACAAACCTAAAGAC GTCTAGGAACCTTTATACAAAGACA

                                        3350                                                3400
              *         *         *         *         *         *         *         *         *         *
TCAAGTATCTTGCTCATGTTCTTAA GCTAGATTTCACACCCGAAATCAA GTGTATGAAGGTATAGATTATGTCT TCATAGTTTTAAGATTTCTCTTTAT
AGTTCATAGAACGAGTACAAGAATT CGATCTAAAGTGTGGGGCTTTAGTT CACATACTTCCATATCTAATACAGA AGTATCAAAATTCTAAAGAGAAATA

                                        3450                                                3500
              *         *         *         *         *         *         *         *         *         *
CTTGTTAAGTTTGGGGAAATTCTTA GAACTTTTTGTGTCCTTTTGTGCTC CAGAAACTATGAACTGGAGTCTCTT AAAGGAATCTTTTCTGAATCTACTT
GAACAATTCAAACCCCTTTAAGAAT CTTGAAAAACACAGGAAAACACGAG GTCTTTGATACTTGACCTCAGAGAA TTTCCTTAGAAAAGACTTAGATGAA
```

FIGURE 3B

```
                                        3550                                        3600
          *         *         *         *         *         *         *         *         *
CTGGTACATCCCACAATCAATGGCT CAGAAACTAGTATAGTAATTTATTT GTGATTTTTAATGTTATGGTTCTGC TTGTAGGCTTCAAGAAAAGTGAATT
GACCATGTAGGGTGTTAGTTACCGA GTCTTTGATCATATCATTAAATAAA CACTAAAAATTACAATACCAAGACG AACATCCGAAGTTCTTTTCACTTAA

                                        3650                                        3700
          *         *         *         *         *         *         *         *         *
TCAAACTTCTAATGAAAGATTATCT ATCAACAATGTGTGCATCCATTGAT GCTGATGAAAAGTCTATCAGTTTGG TAGAATTGCACAAGGACATGCTTAC
AGTTTGAAGATTACTTTCTAATAGA TAGTTGTTACACACGTAGGTAACTA CGACTACTTTTCAGATAGTCAAACC ATCTTAACGTGTTCCTGTACGAATG

                                        3750                                        3800
          *         *         *         *         *         *         *         *         *
TGCTATGAAAGAACTTCTAGTAATG GTAAGTTTTCGGTTACCAGAGTATT AGTGTTTCAATTTTCGGAGCTTTTT GCTCTTACGTTTCTGTTCCTTGTTT
ACGATACTTTCTTGAAGATCATTAC CATTCAAAAGCCAATGGTCTCATAA TCACAAAGTTAAAAGCCTCGAAAAA CGAGAATGCAAAGACAAGGAACAAA

                                        3850                                        3900
          *         *         *         *         *         *         *         *         *
AGATCATGGAGCTTGATACATCAAA GAAGAAAGCTGACTTAGAAGGGATT ACCTCTAGAGGAGATGGCGTAAGGT AATGAGTTTCTGCCTCACTTTTGAA
TCTAGTACCTCGAACTATGTAGTTT CTTCTTTCGACTGAATCTTCCCTAA TGGAGATCTCCTCTACCGCATTCCA TTACTCAAAGACGGAGTGAAAACTT

                                        3950                                        4000
          *         *         *         *         *         *         *         *         *
TTGTGTTTTCTCCATGTTTGTGATC CAATTTGATGCAGGACCCCTGCAAT GGAGATCATTCTCGACGAGCTGACT TATGATGGATACTTGCTGTCAAAAT
AACACAAAAGAGGTACAAACACTAG GTTAAACTACGTCCTGGGGACGTTA CCTCTAGTAAGAGCTGCTCGACTGA ATACTACCTATGAACGACAGTTTTA

                                        4050                                        4100
          *         *         *         *         *         *         *         *         *
TTCTTCAGGTAAGAGACAAACCACT TCTGAAACTCACTGTGGTCTGTAAA ATTCTTCTCTCTGTGGTTCTTGAGT GAAAATTTCTTAATCAGGTTTTCGA
AAGAAGTCCATTCTCTGTTTGGTGA AGACTTTGAGTGACACCAGACATTT TAAGAAGAGAGACACCAAGAACTCA CTTTTAAAGAATTAGTCCAAAAGCT

                                        4150                                        4200
          *         *         *         *         *         *         *         *         *
TGATCCTAAATGGAAGCTAGAGATT GTTCTCCAATACCTTACTAAATACA TTCCTAAGGTTTGTTGTTAATTCTC TGATCTCTCGCTCTCTGATTATCTT
ACTAGGATTTACCTTCGATCTCTAA CAAGAGGTTATGGAATGATTTATGT AAGGATTCCAAACAACAATTAAGAG ACTAGAGAGCGAGAGACTAATAGAA

                                        4250                                        4300
          *         *         *         *         *         *         *         *         *
CTCCAACAAATGATGTACAATATAT AGTTATTTCTCTGATTTCTCTCTCT GACCAAATTTCTTTCATGTTGTAGC CTGTTGTACGTACCCGAAGAACAAC
GAGGTTGTTTACTACATGTTATATA TCAATAAAGAGACTAAAGAGAGAGA CTGGTTTAAAGAAAGTACAACATCG GACAACATGCATGGGCTTCTTGTTG

                                        4350                                        4400
          *         *         *         *         *         *         *         *         *
TGTTCCTCAAGCAGAGGATTCGAAA ACACTAAATGGGATCTTGAAGACGT TTTCAAATGGCACAAATCCAAAGAA CATCACTAAAAAGATAGGACCTGAC
ACAAGGAGTTCGTCTCCTAAGCTTT TGTGATTTACCCTAGAACTTCTGCA AAAGTTTACCGTGTTTAGGTTTCTT GTAGTGATTTTTCTATCCTGGACTG

                                        4450                                        4500
          *         *         *         *         *         *         *         *         *
ATTGTTCAGATCCTCATCGGCCATG CCTTTCTGGTAACACAATAATACCT TTGTTTCTTCTTTCACGTTCTCAGA GCTTTTTTTGGCCTCCTAATGTATT
TAACAAGTCTAGGAGTAGCCGGTAC GGAAAGACCATTGTGTTATTATGGA AACAAAGAAGAAAGTGCAAGAGTCT CGAAAAAAACCGGAGGATTACATAA

                                        4550                                        4600
          *         *         *         *         *         *         *         *         *
TAACTATTCGTCAGGCTCGGCTTAC ATTCTCTGACCCTCACGAAGGAGAC TCTATTTCAGAGATATGCAGTAGTA TCATCTCTGCATTTACTAGTGTAAA
ATTGATAAGCAGTCCGAGCCGAATG TAAGAGACTGGGAGTGCTTCCTCTG AGATAAAGTCTCTATACGTCATCAT AGTAGAGACGTAAATGATCAGATTT

                                        4650                                        4700
          *         *         *         *         *         *         *         *         *
GCGAGTAGATCAGTAAGCTGCAGTT TCTTTCACTCTCAGTTTTTTTTCTC TTTGTGCAATCGAGATACTATACCG CTTTCCTTTGTCCAACCGAAAGTTT
CGCTCATCTAGTCATTCGACGTCAA AGAAAGTGAGAGTCAAAAAAAAGAG AAACACGTTAGCTCTATGATATGGC GAAAGGAAACAGGTTGGCTTTCAAA
```

FIGURE 3B

5 of 5

```
                                           4750                                                                4800
      *          *          *          *          *          *          *          *          *          *
AATGGTTGTTATAGGAAAATCGAGA TTCTACCGTTTGGGAAAGAAGTGTT GTTTACTGCAGGAATGGTACTCAAG GCAAAAGCTTAAAGCATCTAGGAAT
TTACCAACAATATCCTTTTAGCTCT AAGATGGCAAACCCTTTCTTCACAA CAAATGACGTCCTTACCATGAGTTC CGTTTTCGAATTTCGTAGATCCTTA

                                           4850                                                                4900
      *          *          *          *          *          *          *          *          *          *
CAAGATTTTAACAAATCTAGAGACT TCAATTGGATTAGAAGCAAAGAAGT TACAATGTAATGTAGGAATTAAGAG TTACAGATCAATGAATTATGAATTT
GTTCTAAAATTGTTTAGATCTCTGA AGTTAACCTAATCTTCGTTTCTTCA ATGTTACATTACATCCTTAATTCTC AATGTCTAGTTACTTAATACTTAAA

                                           4950                                                                5000
      *          *          *          *          *          *          *          *          *          *
ATATATTCTATAGTTTTGCCAATAC AAAATGAGTGATCTTATGATTCTGT TGATGCCTTTTTCTCACTTATGGAA CATATCAAGACTTGCTTTAGTAACA
TATATAAGATATCAAAACGGTTATG TTTTACTCACTAGAATACTAAGACA ACTACGGAAAAAGAGTGAATACCTT GTATAGTTCTGAACGAAATCATTGT

      *          *          *          *
AATCCATGCCTGATAAGTTTATGCA AAACAATCTTGGCTT
TTAGGTACGGACTATTCAAATACGT TTTGTTAGAACCGAA
```

FIGURE 3B

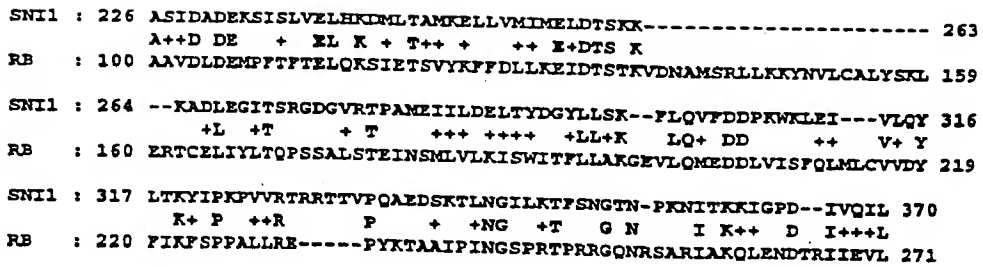

SNI1 : 226 ASIDADEKSISLVELHKDMLTAMKELLVMIMELDTSKK---------- 263
A++D DE + EL K + T++ + ++ E+DTS K
RB : 100 AAVDLDEMPFTFTELQKSIETSVYKFFDLLKEIDTSTKVDNAMSRLLKKYNVLCALYSKL 159
SNI1 : 264 --KADLEGITSRGDGVRTPAMEIILDELTYDGYLLSK--FLQVFDDPKWKLEI---VLQY 316
+L +T + T +++ ++++ +LL+K LQ+ DD ++ V+ Y
RB : 160 ERTCELIYLTQPSSALSTEINSMLVLKISWITFLLAKGEVLQMEDDLVISFQLMLCVVDY 219
SNI1 : 317 LTKYIPKPVVRTRRTTVPQAEDSKTLNGILKTFSNGTN-PKNITKKIGPD--IVQIL 370
K+ P ++R P + +NG +T G N I K++ D I+++L
RB : 220 FIKFSPPALLRE-----PYKTAAIPINGSPRTPRRGQNRSARIAKQLENDTRIIEVL 271

FIGURE 3C

GFP

SNI1-GFP

FIGURE 4

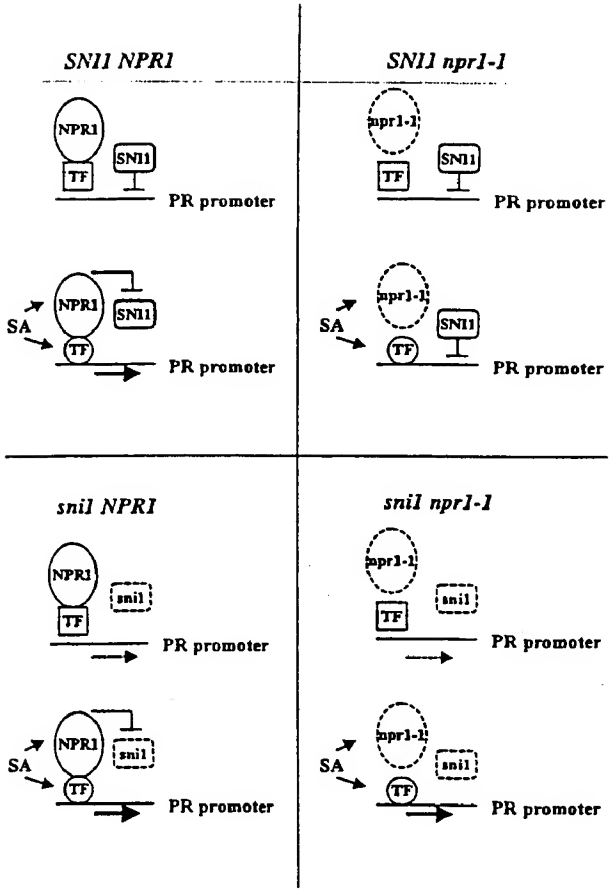

SNI1 NPR1
NPR1
TF
SNI1
PR promoter
SA
SNI1 npr1-1
npr1-1
snil NPR1
snil
snil npr1-1

FIGURE 5

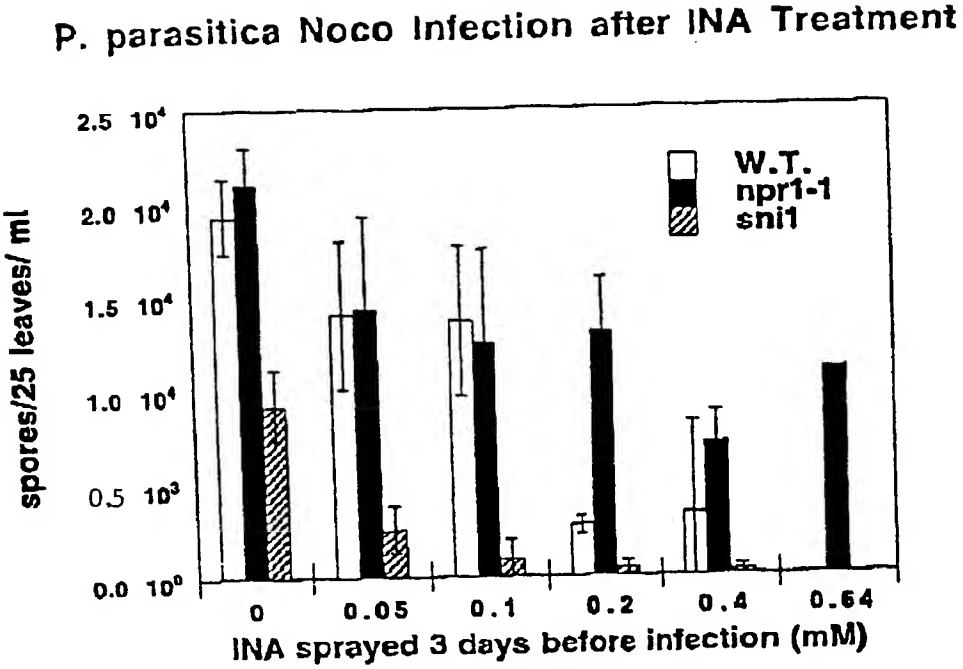

P. parasitica Noco Infection after INA Treatment
spores/25 leaves/ ml
2.5 10⁴
2.0 10⁴
1.5 10⁴
1.0 10⁴
0.5 10³
0.0 10⁰
W.T.
npr1-1
sni1
0
0.05
0.1
0.2
0.4
0.64
INA sprayed 3 days before infection (mM)

FIGURE 6

SEQUENCE LISTING

<110> Duke University

<120> NEGATIVE REGULATORS OF SYSTEMIC ACQUIRED RESISTANCE

<130> 06765/011WO2

<150> 60/142,214
<151> 1999-07-02

<160> 18

<170> FastSEQ for Windows Version 4.0

<210> 1
<211> 1478
<212> DNA
<213> Arabidopsis thaliana

<400> 1
cttctcgttt tcttctttgg tgctgctgag aagaaattag tgaaattgtg aaagagaaga 60
tgtcgaaaga gacgaagggt aataacaata caagcagagt gatgagtggc tatggcggta 120
gcttggaagc taacaccttg gctatgattg attccaccgg agctaaagac agtcgcgacg 180
ctaacgaaga tcgtttgcag tatctggaag ctgttcgtgc tgcttcactt gtacccgaaa 240
atggaattcc tccaaccaac aaaatgtacc aagcgatttt caggatattg agatttggta 300
aaacattgga acttatcaca gcaagtttcc agcttttgac tcaattacat cagcggtttc 360
cttgggttta tgtatctgat tcagctgatc agttggacat cgttgacgag gcttggtcac 420
cgtttaattt cgggtctgat gttgattctg atgaaaagga tttatcagtg agaagcttat 480
ttttgcaaca gctgattcag aacatgaaca aaagagttaa tgagtctgag gaatcagatt 540
taaagatcct tggaaatatg tttctgttca agtatcttgc tcatgttctt aagctagatt 600
tcacaccccg aaatcaagtg tatgaagaaa ctatgaactg gagtctctta aaggaatctt 660
ttctgaatct acttctggct tcaagaaaag tgaatttcaa acttctaatg aaagattatc 720
tatcaacaat gtgtgcatcc attgatgctg atgaaaagtc tatcagtttg gtagaattgc 780
acaaggacat gcttactgct atgaaagaac ttctagtaat gatcatggag cttgatacat 840
caaagaagaa agctgactta gaagggatta cctctagagg agatggcgta aggacccctg 900
caatggagat cattctcgac gagctgactt atgatggata cttgctgtca aaatttcttc 960
aggttttcga tgatcctaaa tggaagctag agattgttct ccaatacctt actaaataca 1020
ttcctaagcc tgttgtacgt acccgaagaa caactgttcc tcaagcagag gattcgaaaa 1080
cactaaatgg gatcttgaag acgttttcaa atggcacaaa tccaaagaac atcactaaaa 1140
agataggacc tgacattgtt cagatcctca tcggccatgc ctttctggct cggcttacat 1200
tctctgaccc tcacgaagga gactctattt cagagatatg cagtagtatc atctctgcat 1260
ttactagtct aaagcgagta gatcagaaaa tcgagattct accgtttggg aaagaagtgt 1320
tgtttactgc aggaatggta ctcaaggcaa aagcttaaag catctaggaa tcaagatttt 1380
aacaaatcta gagacttcaa ttggattaga agcaaagaag ttacaatgta atgtaggaat 1440
taagagttac agatcaatga attatgaatt tatatatt 1478

<210> 2
<211> 432
<212> PRT
<213> Arabidopsis thaliana

<400> 2
Met Ser Lys Glu Thr Lys Gly Asn Asn Asn Thr Ser Arg Val Met Ser
1 5 10 15

```
Gly Tyr Gly Gly Ser Leu Glu Ala Asn Thr Leu Ala Met Ile Asp Ser
            20                  25                  30
Thr Gly Ala Lys Asp Ser Arg Asp Ala Asn Glu Asp Arg Leu Gln Tyr
        35                  40                  45
Leu Glu Ala Val Arg Ala Ala Ser Leu Val Pro Glu Asn Gly Ile Pro
    50                  55                  60
Pro Thr Asn Lys Met Tyr Gln Ala Ile Phe Arg Ile Leu Arg Phe Gly
65                  70                  75                  80
Lys Thr Leu Glu Leu Ile Thr Ala Ser Phe Gln Leu Leu Thr Gln Leu
                85                  90                  95
His Gln Arg Phe Pro Trp Val Tyr Val Ser Asp Ser Ala Asp Gln Leu
            100                 105                 110
Asp Ile Val Asp Glu Ala Trp Ser Pro Phe Asn Phe Gly Ser Asp Val
        115                 120                 125
Asp Ser Asp Glu Lys Asp Leu Ser Val Arg Ser Leu Phe Leu Gln Gln
    130                 135                 140
Leu Ile Gln Asn Met Asn Lys Arg Val Asn Glu Ser Glu Glu Ser Asp
145                 150                 155                 160
Leu Lys Ile Leu Gly Asn Met Phe Leu Phe Lys Tyr Leu Ala His Val
                165                 170                 175
Leu Lys Leu Asp Phe Thr Pro Arg Asn Gln Val Tyr Glu Glu Thr Met
            180                 185                 190
Asn Trp Ser Leu Leu Lys Glu Ser Phe Leu Asn Leu Leu Leu Ala Ser
        195                 200                 205
Arg Lys Val Asn Phe Lys Leu Leu Met Lys Asp Tyr Leu Ser Thr Met
    210                 215                 220
Cys Ala Ser Ile Asp Ala Asp Glu Lys Ser Ile Ser Leu Val Glu Leu
225                 230                 235                 240
His Lys Asp Met Leu Thr Ala Met Lys Glu Leu Leu Val Met Ile Met
                245                 250                 255
Glu Leu Asp Thr Ser Lys Lys Lys Ala Asp Leu Glu Gly Ile Thr Ser
            260                 265                 270
Arg Gly Asp Gly Val Arg Thr Pro Ala Met Glu Ile Ile Leu Asp Glu
        275                 280                 285
Leu Thr Tyr Asp Gly Tyr Leu Leu Ser Lys Phe Leu Gln Val Phe Asp
    290                 295                 300
Asp Pro Lys Trp Lys Leu Glu Ile Val Leu Gln Tyr Leu Thr Lys Tyr
305                 310                 315                 320
Ile Pro Lys Pro Val Val Arg Thr Arg Arg Thr Thr Val Pro Gln Ala
                325                 330                 335
Glu Asp Ser Lys Thr Leu Asn Gly Ile Leu Lys Thr Phe Ser Asn Gly
            340                 345                 350
Thr Asn Pro Lys Asn Ile Thr Lys Lys Ile Gly Pro Asp Ile Val Gln
        355                 360                 365
Ile Leu Ile Gly His Ala Phe Leu Ala Arg Leu Thr Phe Ser Asp Pro
    370                 375                 380
His Glu Gly Asp Ser Ile Ser Glu Ile Cys Ser Ser Ile Ile Ser Ala
385                 390                 395                 400
Phe Thr Ser Leu Lys Arg Val Asp Gln Lys Ile Glu Ile Leu Pro Phe
                405                 410                 415
Gly Lys Glu Val Leu Phe Thr Ala Gly Met Val Leu Lys Ala Lys Ala
            420                 425                 430
```

<210> 3
<211> 5040
<212> DNA
<213> Arabidopsis thaliana

<400> 3

gcttcctctg cagtttggaa cgttccgagc caaaccctaa caccgtttct cgtcgaatcc 60
ctgatttcgg ctgcaaattt cccccacgga cgcttccgta ctcccctgta ttttcttgtt 120
ttgcaagaaa cccgagaaga caaagaacat gaatcaatgg tagggttggt taatggatca 180
tgattggttt tgtttgggaa agaagcaata gcatcgagaa tctctgtcca ttcctggtcc 240
tccgtcgaca tatctaggct ttgaagcatt tctctagaac cttctggtat gtcatcaagg 300
gtaggcaacg cttcccaagt tctagaaact tctggaactt gaattgatct tgtatcggtt 360
tgtggtgttc cgatgtaatt cgcccaaacg ttctttagga tctcgccgtc tagttcttgg 420
atattgccaa aagccattgt tgaatcagag aggagtatgt gttggtgaga tttagggcat 480
tttaaagaga ttgttgcttt gggggttttg tgtgtacgtt gaatgggaaa tctgcggcag 540
gataattggg aagtacagtt ggtgaaactt gtgttctaag gtactgaccg catttcatgg 600
agggttttag gttccacata aaatcagggg ttttctgtgt atacacacac acacgtacca 660
ttatgtgaat agatcatctt aagtcaactt caataaatta atttaataat gttacctcag 720
tttgaagtat aaagattgtg tactattttc attaaaaaag tgcagattgt gtactcttga 780
aaaggactag atgcagagta ttaattcact tttgtataat aaacatatta atcttcagtt 840
taacgcttca tgagaatcta tgacgtgttc ccaccagagg cacaaatccg ttttggagaa 900
ttgtgattat taaatcaaac ataagtttat tgttttaatg tttcgttcca cgctcgtcgc 960
attttaaaaa acttttttaac tatttttttg gttataaaaa gaaatatagc ttctaaatga 1020
cttgtttcta ctgttagatt gatgaacctt aatactgatt tagcttttca tacatcgata 1080
aagaattctt cggtgtttag ttaagtttgt tagaagttat tattttacca tacgatcaca 1140
gtttaacgca tattgcctag agaatataat ataatgataa ctaaacgcta tcaaaatgtg 1200
aaacgaaata agaaaaaaca tgtaagggct aagtcatcac taacttttta tccaagtatt 1260
atttgtatga gatcattttc cgtaagtcca aagatttgaa acaatgatga gaggtgaata 1320
gaatatataa attccacacg ctttgaaaca tagcttacaa ttgaaaaaaa atgaaaaaga 1380
gattaatatc tctattcaat gtattataga gaatgagcta gagatacttt atatggtagg 1440
ttcaagtcac ctcaagtacg tatcagctaa tattaaaaaa ctatctgcta atttttatat 1500
caatcgttta agattcattc attcgttctc cactacgaga gtttcttgct ttggcacata 1560
tatagttcaa gaaagtctcg tgttggagag actccaagcg tgctagagcg tttcgattag 1620
aaaggtgttt tgttcgagca ttaaagattg ttgcaatggt tctatcttaa ggtagtcatt 1680
tgttaaattt tggtattcaa tctcgagagc ttcataagtc tagcgagctt cataaacaca 1740
ctacaagtca tgtgtataaa atttccaatt ctgtaatcac aagtctaaac ttaatctgaa 1800
tccaaatcac aaaaagcatt ttcaaaattg attaaaaaaa aaactacaat atagaaaaga 1860
ttttaattga tgcttggaac ggaactaaca ttacctgtta tgatttcggg cctgaaagta 1920
ttctgagttt tgattcagaa ccactcggta ttaaagaaaa agaagactta ctacaaaaaa 1980
aaaattatat atatttttat gaattattat aaccaactaa tattgtctct attggttcta 2040
agttcaaact tctaaccttc gagggccggg ttcaaattcc agtaagtgat ttatttttg 2100
ctaacaattc tcttatggcg ggaaaattga cttctgtcga gaattagggt ttctacaaat 2160
ctttctccgt tctaatttcg gattaagatt tcaatctctc cttctcgttt tcttctttgg 2220
tgctgctgag aagaaattag tgaaattgtg aaagagaaga tgtcgaaaga gacgaagggt 2280
aataacaata caagcagagt gatgagtggc tatggcggta gcttggaagc taacaccttg 2340
gctatgattg attccaccgg agctaaagac agtcgcgacg ctaacgaaga tcgtacgttt 2400
cctcaatttt caatcttcgt ctttgttttt gttaattgta ttgatccttt gaatgaaaaa 2460
ccttaggttt gcagtatctg gaagctgttc gtgctgcttc acttgtaccc gaaaatggaa 2520
ttcctccaac caagtaaaaa ataaaaaact ctgtttttct aagaaatttg aagtttttac 2580
ctctaaaaga gtactgattt tgatttgggt tttgtttaat tttgaagcaa aatgtaccaa 2640
gcgattttca ggatattgag atttggtaaa acattggaac ttatcacagc aagtttccag 2700
cttttgactc aattacatca ggtacttgtt gcaagatttt ggtagtgtaa tttgtccaat 2760
aacttttatg aggtttcagt gttttgctta ttatgattat gttttgtttc agcggtttcc 2820
ttgggtttat gtatctgatt cagctgatca gttggacatc gttgacgagg tacttgttac 2880
aaaagttttg atgtgttttt gttcattctt tgttatggtg gggatggttg atttcatgtg 2940
tttgtttatg gttttgtttt gcaggcttgg tcaccgttta atttcgggtc tgatgttgat 3000
tctgatgaaa aggatttatc agtgagaagc ttatgtaagg acgtttgtct tctcttttg 3060
agttagcttt ttgttggttc ttgctaaata tgatttttg tttgttttat gatttcagtt 3120
ttgcaacagc tgattcagaa catgaacaaa agagttaatg agtctgagga atcagattta 3180
aaggttgatt tatttgttat gttgcagata agctttaaaa attggtagac tttcacacaa 3240
cttgttgtta tttgattctt ttgtttggat ttctgcagat ccttggaaat atgtttctgt 3300

tcaagtatct tgctcatgtt cttaagctag atttcacacc ccgaaatcaa gtgtatgaag 3360
gtatagatta tgtcttcata gttttaagat ttctctttat cttgttaagt ttggggaaat 3420
tcttagaact ttttgtgtcc ttttgtgctc cagaaactat gaactggagt ctcttaaagg 3480
aatcttttct gaatctactt ctggtacatc ccacaatcaa tggctcagaa actagtatag 3540
taatttattt gtgattttta atgttatggt tctgcttgta ggcttcaaga aaagtgaatt 3600
tcaaacttct aatgaaagat tatctatcaa caatgtgtgc atccattgat gctgatgaaa 3660
agtctatcag tttggtagaa ttgcacaagg acatgcttac tgctatgaaa gaacttctag 3720
taatggtaag ttttcggtta ccagagtatt agtgtttcaa ttttcggagc tttttgctct 3780
tacgtttctg ttccttgttt agatcatgga gcttgataca tcaaagaaga aagctgactt 3840
agaagggatt acctctagag gagatggcgt aaggtaatga gtttctgcct cacttttgaa 3900
ttgtgttttc tccatgtttg tgatccaatt tgatgcagga cccctgcaat ggagatcatt 3960
ctcgacgagc tgacttatga tggatacttg ctgtcaaaat ttcttcaggt aagagacaaa 4020
ccacttctga aactcactgt ggtctgtaaa attcttctct ctgtggttct tgagtgaaaa 4080
tttcttaatc aggttttcga tgatcctaaa tggaagctag agattgttct ccaatacctt 4140
actaaataca ttcctaaggt ttgttgttaa ttctctgatc tctcgctctc tgattatctt 4200
ctccaacaaa tgatgtacaa tatatagtta tttctctgat ttctctctct gaccaaattt 4260
ctttcatgtt gtagcctgtt gtacgtaccc gaagaacaac tgttcctcaa gcagaggatt 4320
cgaaaacact aaatgggatc ttgaagacgt tttcaaatgg cacaaatcca aagaacatca 4380
ctaaaaagat aggacctgac attgttcaga tcctcatcgg ccatgccttt ctggtaacac 4440
aataatacct ttgtttcttc tttcacgttc tcagagcttt ttttggcctc ctaatgtatt 4500
taactattcg tcaggctcgg cttacattct ctgaccctca cgaaggagac tctatttcag 4560
agatatgcag tagtatcatc tctgcattta ctagtctaaa gcgagtagat cagtaagctg 4620
cagtttcttt cactctcagt tttttttctc tttgtgcaat cgagatacta taccgctttc 4680
ctttgtccaa ccgaaagttt aatggttgtt ataggaaaat cgagattcta ccgtttggga 4740
aagaagtgtt gtttactgca ggaatggtac tcaaggcaaa agcttaaagc atctaggaat 4800
caagatttta acaaatctag agacttcaat tggattagaa gcaaagaagt tacaatgtaa 4860
tgtaggaatt aagagttaca gatcaatgaa ttatgaattt atatattcta tagttttgcc 4920
aatacaaaat gagtgatctt atgattctgt tgatgccttt ttctcactta tggaacatat 4980
caagacttgc tttagtaaca aatccatgcc tgataagttt atgcaaaaca atcttggctt 5040

<210> 4
<211> 145
<212> PRT
<213> Arabidopsis thaliana

<400> 4
Ala Ser Ile Asp Ala Asp Glu Lys Ser Ile Ser Leu Val Glu Leu His
1 5 10 15
Lys Asp Met Leu Thr Ala Met Lys Glu Leu Leu Val Met Ile Met Glu
20 25 30
Leu Asp Thr Ser Lys Lys Lys Ala Asp Leu Glu Gly Ile Thr Ser Arg
35 40 45
Gly Asp Gly Val Arg Thr Pro Ala Met Glu Ile Ile Leu Asp Glu Leu
50 55 60
Thr Tyr Asp Gly Tyr Leu Leu Ser Lys Phe Leu Gln Val Phe Asp Asp
65 70 75 80
Pro Lys Trp Lys Leu Glu Ile Val Leu Gln Tyr Leu Thr Lys Tyr Ile
85 90 95
Pro Lys Pro Val Val Arg Thr Arg Arg Thr Thr Val Pro Gln Ala Glu
100 105 110
Asp Ser Lys Thr Leu Asn Gly Ile Leu Lys Thr Phe Ser Asn Gly Thr
115 120 125
Asn Pro Lys Asn Ile Thr Lys Lys Ile Gly Pro Asp Ile Val Gln Ile
130 135 140
Leu
145

<210> 5
<211> 172
<212> PRT
<213> Homo Sapiens

<400> 5
Ala Ala Val Asp Leu Asp Glu Met Pro Phe Thr Phe Thr Glu Leu Gln
1 5 10 15
Lys Ser Ile Glu Thr Ser Val Tyr Lys Phe Phe Asp Leu Leu Lys Glu
20 25 30
Ile Asp Thr Ser Thr Lys Val Asp Asn Ala Met Ser Arg Leu Leu Lys
35 40 45
Lys Tyr Asn Val Leu Cys Ala Leu Tyr Ser Lys Leu Glu Arg Thr Cys
50 55 60
Glu Leu Ile Tyr Leu Thr Gln Pro Ser Ser Ala Leu Ser Thr Glu Ile
65 70 75 80
Asn Ser Met Leu Val Leu Lys Ile Ser Trp Ile Thr Phe Leu Leu Ala
85 90 95
Lys Gly Glu Val Leu Gln Met Glu Asp Asp Leu Val Ile Ser Phe Gln
100 105 110
Leu Met Leu Cys Val Val Asp Tyr Phe Ile Lys Phe Ser Pro Pro Ala
115 120 125
Leu Leu Arg Glu Pro Tyr Lys Thr Ala Ala Ile Pro Ile Asn Gly Ser
130 135 140
Pro Arg Thr Pro Arg Arg Gly Gln Asn Arg Ser Ala Arg Ile Ala Lys
145 150 155 160
Gln Leu Glu Asn Asp Thr Arg Ile Ile Glu Val Leu
165 170

<210> 6
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Based on Arabidopsis thaliana

<400> 6
gaagctattg gatagatg 18

<210> 7
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Based on Arabidopsis thaliana

<400> 7
gttgagcaag tgcaact 17

<210> 8
<211> 16
<212> DNA
<213> Artificial Sequence

<220>
<223> Based on Arabidopsis thaliana

<400> 8
ctggcatccg tgaaac 16

<210> 9
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Based on Arabidopsis thaliana

<400> 9
gcaggacttg atgtatcc 18

<210> 10
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Based on Arabidopsis thaliana

<400> 10
ccatccaaag gcgataac 18

<210> 11
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Based on Arabidopsis thaliana

<400> 11
ccaaactact accggatg 18

<210> 12
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Based on Arabidopsis thaliana

<400> 12
gtgatggtga gggcttc 17

<210> 13
<211> 16
<212> DNA
<213> Artificial Sequence

<220>
<223> Based on Arabidopsis thaliana

<400> 13
cgtcgggatc tacagg 16

<210> 14
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Based on Arabidopsis thaliana

<400> 14
gatggcaatt gctggag 17

<210> 15
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Based on Arabidopsis thaliana

<400> 15
ctaatgggat gcgactc 17

<210> 16
<211> 16
<212> DNA
<213> Artificial Sequence

<220>
<223> Based on Arabidopsis thaliana

<400> 16
gatgagatgt gctgag 16

<210> 17
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Based on Arabidopsis thaliana

<400> 17
catcgatttc gccgttc 17

<210> 18
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Based on Arabidopsis thaliana

<400> 18

```
Cys Phe Ser Asp Pro His Glu Gly Asp Ser Ile Ser Glu
 1               5                  10
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/18270

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12N 15/29, 15/82; A01H 5/00, 5/10
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/278, 279, 286, 295, 298; 435/69.1, 320.1, 419, 468; 536/23.1, 23.6, 24.1, 24.5; 530/370, 387.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98/04586 (JOHN INNES CENTRE INNOVATIONS LIMITED) 05 February 1998 (05.02.98) see pages 1, 5, 37, 47-49	1-27, 35-54
Y	UKNES et al. Acquired Resistance in Arabidopsis. The Plant Cell. June 1992, Vol. 4, pages 645-656, see pages 645-647, 649-650.	59-68
Y	RYALS et al. The Arabidopsis NIMI Protein Shows Homology to the Mammalian Transcription Factor Inhibitor IkB. The Plant Cell. March 1997, Vol. 9, Pages 425-439, see pages 426, 430, 435-436.	9-15, 23-24, 36-38

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 OCTOBER 2000	17 NOV 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT	Authorized officer Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/18270

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	YALPANI et al. Salicylic Acid is a Systemic Signal and an Inducer of Pathogenesis-Related Proteins in Virus-Infected Tobacco. The Plant Cell. August 1991, Vol. 3, pages 809-818, see pages 811-810 and 816.	28-34
Y, P	US 5,986, 082 A (UKNES et al) 16 November 1999 (16.11.99) see entire document, especially columns 24-32.	28-34

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/18270

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

800/278, 279, 286, 295, 298; 435/69.1, 320.1, 419, 468; 536/23.1, 23.6, 24.1, 24.5; 530/370, 387.1

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

STN/CAS, WEST2.0
search terms: negative r`gulator, SAR, signal components, SA, INA BTH, mutant or transgenic plant, increased resistance, pathogen resistance, broad-spectrum